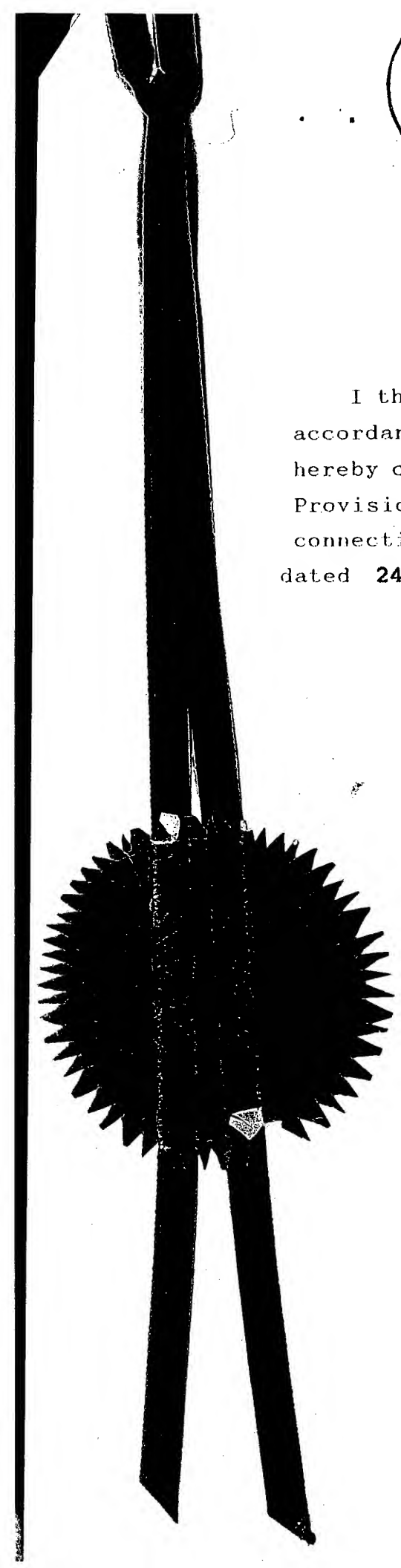

GOVERNMENT OF INDIA
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I the undersigned being an Officer duly authorised in accordance with the provision of the Patents Act, 1970 hereby certify that annexed hereto is the photocopy of the Provisional Specification and Drawing Sheets filed in connection with Application for Patent No. **3825/Del/98** dated **24.12.1998.**

Witness my hand this **24th** day of **April,** 2000.

(H.C.BAKSHI)
Dy.Controller of Patents and Designs.

FORM 3
THE PATENTS ACT, 1970

PROVISIONAL SPECIFICATION
(See Section 10)

A PROCESS FOR THE PREPARATION OF CLOT- SPECIFIC STREPTOKINASES POSSESSING USEFUL PLASMINOGEN ACTIVATION CHARACTERISTICS.

ORIGINAL

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH
RAFI MARG, NEW DELHI - 110 001, INDIA
AN INDIAN REGISTERED BODY
INCORPORATED UNDER THE
REGISTRATION OF SOCIETIES ACT
(Act XXI of 1860)

THE FOLLOWING SPECIFICATION PARTICULARLY DESCRIBES AND ASCERTAINS THE NATURE OF THIS INVENTION AND THE MANNER IN WHICH IT IS TO BE PERFORMED.

The present invention relates to a process for the production of chimeric streptokinases with enhanced fibrin selectivity as well as kinetics of plasminogen activation that are distinct from that of natural streptokinase in being characterized by a delay in the initial rate in the catalytic conversion of plasminogen to plasmin in the presence of a large excess of the former. Thus, PG activation by these altered forms, or analogs, of streptokinase is characterized by an initial lag of several minutes during which there is little plasminogen activation. After the lag period, a rapid burst of plasmin formation occurs whence their plasminogen activation rates become similar to that observed in case of native i.e. unmodified SK. The presence of these two properties in these chimeric proteins together (i.e. fibrin affinity and lag in plasminogen activation), therefore, enables them to bind tightly with fibrin, the proteinaceous substance of blood clots subsequent to their introduction into the vascular system, thus allowing their rapid localization to the site of the pathological thrombus. However, as soon as the plasminogen activation lag is overcome, they can activate the plasminogen in the immediate vicinity of the thrombus, thereby obviating the systemic PG activation frequently encountered during clinical use of streptokinase. These chimeric proteins can be used for thrombolytic therapy for various kinds of cardiovascular disorders. By the usage of the term Streptokinase herein is meant the protein encoded by *Streptococcus* species that shows the biological property of plasminogen activation, and used in a generic sense (unless specified otherwise) to encompass streptokinase/s produced either naturally from various species of *Streptococcus*, or those produced in heterologous systems by recombinant DNA methods.

In recent years, thrombolytic therapy with fibrinolytic agents, such as Streptokinase (SK), tissue plasminogen activator (TPA) or urokinase (UK) has revolutionized the clinical management of diverse circulatory diseases e.g., deep-vein thrombosis, pulmonary embolism and myocardial infarction. These agents exert their fibrinolytic effects through activation of plasminogen (PG) in the circulation by cleavage of the scissile peptide bond between residues 560 and 561 in PG. As a result, the inactive zymogen is transformed to its active form, the serine protease plasmin (PN), which then acts on fibrin to degrade the latter into soluble degradation products. It may be mentioned here that PN, by itself, is incapable of activating PG to PN; this reaction is catalyzed by highly specific proteases like TPA and UK, which possess an unusually narrow protein substrate preferance, namely a propensity to cleave the scissile peptide bond in PG. However, unlike UK and TPA, SK has no proteolytic activity of its own, and it activates PG to PN indirectly by first forming a high-affinity equimolar complex with PG, known as the activator complex (reviewed in Castellino, F.J., 1981, *Chem. Rev.* 81: 431).

Of the several thrombolytic agents used clinically, SK is probably one of the most-often employed, particularly because of its markedly lower cost when compared to TPA and UK. However, the choice of thrombolytic agent during therapy is dictated by a number of factors besides cost, such as the presence of side-effects and their severity, *in vivo* metabolic survival of the drug (e.g., circulatory clearance rates), fibrin selectivity and/or affinity, immunological reactivity etc. SK is a highly potent PG activator, and has a relatively long plasma half-life which, together, impart a certain advantage to this drug as compared to its counterparts viz., TPA and

UK. However, due to a lack of any appreciable fibrin clot-specificity in the former, the administration of therapeutically effective doses of SK often results in systemic PG activation (as opposed to the clinically desired scenario wherein PG activation remains essentially limited to the locale of the thrombus intended to be dissolved) resulting in hemorrhagic complications due to the proteolytic degradation of blood factors by the plasmin generated throughout the circulatory system. In contrast, TPA shows a strong affinity as well as selectivity for fibrin, a property that automatically confers on TPA a distinct advantage in fibrinolytic therapy as compared to the other clot-dissolver drugs. TPA has an intrinsic affinity for fibrin due to the presence of autonomously folded units (domains) in its polypeptide structure that are specific for binding with fibrin (Banyai, L., Varadi, A., and Patthy, L., 1983, *FEBS Lett.* 163: 37). As a result, TPA can potentially "home" onto its target (fibrin clot) in the presence of a large excess of a closely homologous protein in vascular system viz., fibrinogen. In addition, TPA displays another advantageous property i.e. upon binding to fibrin, its ability to activate PG is considerably enhanced, ensuring that a more localized PG activation is favoured over a systemic one due to a lowered rate of free plasmin generation in the circulation. Clearly, it follows from these considerations that if a fibrin affinity/selectivity could be integrated into SK, a molecule which otherwise possesses little fibrin affinity of its own, it would considerably enhance the therapeutic efficacy of this thrombolytic agent. With respect to the other coveted trait in a fibrinolytic agent, such as that described above for TPA above (viz., considerably lowered activity while circulating in the vascular system but enhanced PG activating ability in the presence of fibrin), attempts have been made in the past to produce analogs of SK with greater circulatory half-lives and decreased systemic plasmin generation by incorporating properties such as a slower rate of PG activation into the fibrinolytic agent. One example where this has been successfully accomplished is that of anisoylated streptokinase plasmin activator complex, abbreviated APSAC (sold under the trade-name 'Eminase' by the Beecham pharmaceutical group) (reference: Smith, R.A.G., Dupe, R.J., English, P.D., andGreen, J.,1981, *Nature* 290:505) in which the catalytically important serine residue of the plasmin component is blocked by reversible acylation. The generalized plasmin activation coincident with the administration of unmodified SK has been reported to be appreciably diminished during thrombolytic therapy with APSAC since the deacylation of the covalently modified serine in the SK-acylated plasmin complex occurs slowly in the vascular system.

It is thus generally recognised that it will be of significant clinical advantage if SK could be engineered to possess increased fibrin affinity/specificity together with a markedly slower initial rate of activation of PG. Thus, soon after injection into the body, whilst it is still in an inactive or partially active state it will bind to the pathological fibrin clot during its sojourn through the vascular system in an inactive/partially active state. However, after an initial lag (a property engineered into the derivative/analog) it will become fully activated in the immediate vicinity of the clot, thereby obviating the systemic PG activation coincident with natural SK administration. Whilst the former property would be expected to confer on the thrombolytic agent an ability to target itself to the immediate locale of the pathological clot and thus help build up therapeutically

effective concentrations of the activator therein, the initially slow kinetics of PG activation would result in an overall diminished generation of free plasmin in the circulation. The net result shall be a continued and more efficient fibrinolysis at the target sustained by considerably lowered therapeutically effective dosages of the thrombolytic agent.

In the past, the gene encoding for SK has been isolated from its natural source (*Streptococcus* species) and cloned into several heterologous micro-organisms such as yeast (Hagenson, M.J., Holden, K.A., Parker, K.A., Wood,P.J., Cruze, J.A., Fuke. M., Hopkins, T.R., Stroman, D.W.,1989, *Enzyme. Microb.Technol.* 11:650), bacteria viz., *E. coli*, alternate species of *Streptococcus* (Malke, H., Gerlach, D., Kohler, W., Ferretti, J.J., 1984, *Mol.Gen.Genet.* 196:360), and *Bacillus* (Wong, S.L., Ye, R.., Nathoo S.,1994, *Applied and Env. Microbiol.* 1:517). In addition, hybrid SK derivatives with "Kringle" type fibrin binding domains derived from PG, and methods of obtaining the same by rDNA techniques, have been described (EU 0397 366 A1). However, five such Kringle regions are *already* present in the natural SK-PG activator complex, being an integral part of PG in the activator complex. Hence, there is a need to impart a qualitatively different fibrin-affinity and/or specificity to the activator complex, particularly of a type associated with TPA, a very effective thrombolytic agent possessing much greater fibrin affinity than SK. TPA is known to contain a fibrin-associating "finger" domain, which is structurally and functionally very similar to the fibrin-binding domains present in fibronectin, a multi-functional protein with ability to interact with a number of other proteins besides fibrin e.g., collagen, heparin, actin etc (reviewed in Ruoslahti, E., 1988, *Ann. Rev. Biochem.* 57:375). Methods for the imaging of fibrin-containing substances, such as pathological clots and/or atherosclerotic plaques *in vivo* by using large radio-labeled polypeptides derived from fibronectin, and bearing these FBDs have been disclosed (see: PCT WO 91/17765); this patent also discloses chemically cross-linked FBD-containing polypeptides and a thrombolytic agent (SK) to effect thrombus-targeted fibrinolysis. The cross-linking procedure resulted in the generation of a complex mixture of heterogenously cross-linked molecules with variable FBD and SK content, since the bifunctional agents essentially cross-linked any of the large number of lysine side-chains present in the participating molecules viz. SK and HPG likely generating both dimers and multimers with both homo- (e.g., SK-SK or FBD-FBD types) and hetero-crosslinked molecules. However, it is noteworthy that the SK molecules chemically cross-linked with fibrin binding polypeptides showed an overall level of PG activator activity roughly comparable to that of unmodified SK, and no alteration was observed in the rate of PG activation or the presence of an initial lag in the PG activation kinetics.

In contrast, an important attribute of the embodiment of the present invention is the introduction of an initial period of lag in the rate of PG activation in the modified forms of SK which have been designed using precisely defined elements of the human FN protein and SK resulting in the conferrment of both fibrin selectivity and altered activation of PG simultaneously into the resultant constructs.

The molecular basis for the fibrin affinity displayed by fibronectin has been studied in some detail in recent years (Matsuka, Y.V., Medved, L.V., Brew, S.A. and Ingham, K.C., 1994, J. Biol. Chem. 269:9539). Under physiological conditions, FN first interacts reversibly (but with relatively high affinity) with fibrin and is then covalently incorporated into the fibrin clot matrix through clotting factor XIII, a transglutaminase (reviewed in: Ruoslahti, E., 1988, *Ann. Rev. Biochem.* 57:375), whose action results in the covalent cross-linking between FN and a lys residue in fibrin(ogen) at the reactive Gln (residue 3) of the former. The region/s responsible for the interaction of FN with fibrin have been identified to reside both in the N-terminal as well as the C-terminal ends of this multi-domain protein. The N-terminal region of FN comprises of five finger modules (FBDs) as well as a transglutaminase cross-linking (TG) site, whereas the C-terminal region, lacking a TG site, contains three modules, as demonstrated by the binding of different polypeptides derived from FN carrying these two broad regions to fibrin-agarose. The exact domains in the N-terminal region responsible for the avid binding of the FN module, and their relative contributions towards this interaction have been analysed closely (Matsuka, Y.V., Medved, L.V., Brew, S.A. and Ingham, K.C., 1994, *J. Biol. Chem.* 269:9539 and Rostagno et al., 1994; *J. Biol. Chem.* 269: 31938) by expressing DNA segments encoding various combinations of the modules in heterologous cells and/or by examining the fibrin binding properties of polypeptide fragments carrying these modules prepared by limited proteolysis of FN. These studies clearly identified that of all the individual modules present in the N-terminal region of FN, the bi-modular arrangement viz., FBD 4 and 5 domains, displayed a fibrin affinity significantly comparable to the interaction of the full-length FN molecule, in contrast to all the other domains either as pairs or individually (including 4 and 5) which displayed poor affinity at 37 °C. It is therefore clear from these studies that physiologically effective fibrin binding is not a common property of all the modules, either individually or in pairs, but is principally located in the FBD pair of 4 and 5.

The main objective of the present invention is to provide a process for the preparation of chimeric streptokinase-based plasminogen activator molecules with improved characteristics viz., improved fibrin affinity and plasminogen activation kinetics with initial lag of several minutes.

Another objective of the process of the present invention is to provide a genetic system for the production of the hybrid polypeptides, which includes DNA segments encoding the polypeptides, plasmids containing these genetic elements capable of their expression into protein, as well as microorganisms transformed with these plasmids.

Yet another objective of the process of the present invention is to design a process for the production of the hybrid plasminogen activator molecules in pure form from cultures of the microorganisms harbouring the expression plasmids.

The process of the invention may be performed by conventional recombinant DNA techniques e.g., those described in 'Sambrook et al., Molecular Cloning: A Laboratory Manual'

(IInd Ed., Cold Spring Harbor Press, 1989) and 'DNA Cloning' (vol. I to III) (Glover, D.M., [Ed.], IRL Press Ltd., London, 1987), among several other manuals/compendia of protocols, and the techniques of protein purification and characterization, in particular the various chromatographic methods employed conventionally for purification and downstream processing of natural and recombinant proteins and enzymes viz., hydrophobic interaction chromatography (HIC), ion-exchange and gel filtration chromatographies, and affinity chromatographic techniques well-known in the field of protein biochemistry (e.g., in this regard, reference may be made to : (i) Protein purification. Principles, high resolution methods and applications. Janson, J-C., and Ryden, L., [Ed.], VCH Publishers Inc., New York, 1989; (ii) Process Chromatography: A practical guide. Sofer, G.K., and Nystrom, L. E., [Ed.], Academic Press, New York, 1989).

Accordingly, the present invention provides a process for the preparation of novel chimeric streptokinases prepared through hybrid formation with selected fibrin binding domains derived from other suitable proteins which comprises :

(a) Preparing a replicable expression vector capable, in a suitable host cell, of intracellular hyper-expression of DNA encoding a nucleotide sequence for SK (or a modified form of SK), prepared biochemically or chemically, or through a combination of the two methods *in vitro* or *in vivo*, as appropriate,

(b) Preparing another DNA polymer by biochemical or chemical means or appropriate combination thereof, that encodes for the fibrin binding domains of selected natural proteins, such as human or animal fibronectin or TPA that are resposible for conferring affinity and/or specificity for fibrin, and linking it to a replicable plasmid that is capable of undergoing multiplication in a suitable host cell,

(c) Construction of hybrid genes, using conventional methods of recombinant DNA technology, between the DNA encoding for SK, or appropriate parts thereof, with the DNA encoding for selected fibrin binding domains derived from the human fibronectin cDNA in the same (i.e. native) translational frame, and ligation of hybrid genes into a plasmid vehicle,

(d) Introducing the plasmid construct containing the hybrid gene obtained at step (c) into an appropriate host, such as *E. coli*, *Bacillus sp.*, yeast etc,

(e) Culturing the host cells harboring the expression plasmid bearing the SK-FBD chimeric gene in an appropriate fermentation medium,

(f) Removing the cells by centrifugation, filtration and the like, and lysing the cells by conventional procedures, such as sonication, chemical or mechanical lysis, and removal of unlysed cells, cellular debris etc, and followed by partially purifying the expressed chimeric polypeptides using

conventional procedures such as precipitation with salts, or organic solvents, or chromatography on suitable media, or a combination of these methods,

(g) Refolding the hybrid polypeptide to a biologically active and structurally intact form,

(h) Purifying the biologically active hybrid polypeptide from the crude, cell-free lysate obtained at step (f) or (g) after refolding, by conventional methods of protein purification.

The details of the present invention are:

The present invention is based on a number of basic principles inbuilt into the design of the SK-FBD chimeras and considered vital to achieve the simultaneous incorporation of the desired properties of fibrin affinity and altered kinetics of activation of PG into the constructs. By the latter property is meant that the virtually immediate PG activation displayed by SK upon addition to excess of the latter molecules is so altered that for at least several minutes post-addition to PG, there is a lag, or delay (wherein little PG activation is observed) in the initiation of the PG activation reaction catalysed by the engineered activator protein. The direct implication of this property is that once injected into the body, the protein could then traverse in an inactive state through the circulatory system and bind to the pathological clot by virtue of its fibrin affinity thereby obviating or minimizing sytemic PG activation. To achieve this 'functional objective', the design utilizes the fusion of 'minimal' regions of the FBDs of human fibronectin (or its homologous sequences present in other proteins) with SK (or its partially truncated forms) at strategically useful points so as to kinetically hinder the initial interaction of SK with PG necessary to form the 1:1 stoichiometric activator complex. It is known that of the 414 residues constituting native SK, only the first 15 residues and the last 31 residues are expendable, with the resultant truncated polypeptide being nearly as active as the native full-length protein in terms of PG activation ability (Jackson, K.W., and Tang, J. (1982) *Biochemistry 21:6620*). Further truncation at either end results in drastic decrease in the activity associated with the molecule (Malke, H., Roe, B., and Ferretti, J.J. (1987) In: *Streptococcal Genetics*. Ferretti, J.J., and Curtis, R. III [Ed.]Proc. American Society for Microbiology., Wash. D.C. p. 143). It has been demonstrated that SK interacts with PG through at least two major loci, mapped between residues 16-51 and 230-290 (Nihalani, D., Raghava, G.P.S., and Sahni, G., 1997, *Prot. Sci.* 6:1284), and probably also the region in and around residues 331-332 (Lin, F.L., Ocun, S., Houng, A., and Reed, G.L., 1996, *Biochemistry* 35:16879); in addition, the sequences at the C-terminal ends, especially before the last 30-32 residues of the native sequence (Kim, I.C., Kim, J.S., Lee, S.H., and Byun, S.M., 1996, *Biochem. Mol. Bio. Int.* 40:939. Lee, S.H., Jeong, S.T., Kim, I.C., and Byun, S.M., 1997, *Biochem. Mol. Bio. Int.* 41:199. Fay, W.P., Bokka, L.V., 1998, *Thromb. Haemost.* 79:985) are important in generating the activator activity associated with the complex. Since a primary consideration in designing the SK-FBD chimeras was the the engineering of a decreased, or kinetically slowed, initial PG activation rate, we reasoned that either the C- or N-termini (or both, together) could be utilized to bear the

FBDs in the hybrid structures, and that the presence of such 'extra' domains in SK, either full-length or already truncted to the 'most permissible limits' would suitably retard and/or delay the PG activation rates observed normally with native SK and PG. If the polypeptide in between these two distinct parts constituting the chimera were sufficiently flexible, proteolytic scission in this region would then result in the removal of the retarding portion from the SK and lead to a burst of PG activation after an initial delay. As discussed above, if the thrombolytic agent traverses the circulation prior to this activation, the fibrin affinity in the chimera would allow it to bind to the clot, thereby localizing the PG activation it to the immediate vicinity of the thrombus.

The amino acid sequence of human FN is known to be composed of three types of homologous repeats (termed type-1, type-2 and type-3), of which the FBDs at the amino terminus of FN are made of five type-1 repeats, each approximately 50 residues long and containing two disulfide bridges. The C-terminus of FN also has three type-2 homology repeats that are involved in fibrin-FN interactions. Therefore, altogether, a large portion of the FN molecule, representing the several N- and C-terminally located FBDs, could be linked with SK if all of the fibrin interacting regions need to be incorporated into the contemplated SK-FBD chimeras. However, such a design produces a chimeric protein that is not only too bulky, but also decreases the probability for the polypeptide to fold into a biologically active conformation due to the presence of a large number of S-S bridges that may form non-native, intra- and inter-molecular disulfide bonds. Instead, a potentially more worthwhile proposition is to seek miniaturised but, nevertheless, functionally active combinations of selectively truncated regions of SK and/or FBDs.

Accordingly, in a preferred embodiment, the present invention discloses a design of SK-FBD chimera that utilises the translational in-frame fusion of the DNAs encoding SK and the minimally essential parts of the FN gene that are capable of possessing significant fibrin affinity on their own.

In another preferred embodiment, the FBDs are fused in-frame at the N-terminal end of the SK. Once expressed into protein and refolded oxidatively to form the correct (i.e. native-like) disulfide bonds, the desired characteristic in the chimera viz., characteristic PG activation properties characterized by an initial lag in the PG activation rates are seen, together with significant fibrin affinity.

In yet another embodiment, the SK portion, devoid of its 31 C-terminal amino acid residues, is fused through polypeptide linkages with the N-terminal end of two of the FBDs (4 and 5 domains), shown to possess independent fibrin binding capability, through a short 'linker' region comprising of a relatively small stretch of amino acid sequence that is not conformationally rigid but is flexible. Thus, a hybrid SK-FBD is generated that contains a C-terminally truncated SK portion and the most important relevant regions of the FN gene with respect to fibrin interactions.

In yet another embodiment, FBDs are fused at both the ends of SK (in the configuration represented as 'FBD-SK-FBD') to achieve the desired functionality in the hybrid construct viz., PG activation as well as fibrin selectivity.

The constructions described above, and schematically depicted in Fig. 1, can be made utilising rDNA and selective DNA amplification techniques (e.g., the well-known polymerase chain reaction technique, abbreviated PCR) (reference, in this regard may be made to: Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H. A., and Arnheim, N., 1985, *Science* 230: 1350; Mullis, K.B., and Faloona, F., 1987, *Methods in Enzymol.* 155:335). The hybrid genes are then expressed in heterologous hosts such as bacteria (e.g. *E. coli*), or other suitable organisms, to obtain the chimeric polypeptides. These are then purified to render them substantially free of other components derived from the host producer cells. In case the polypeptide is expressed in a host system not capable of efficient re-oxidative folding of the primary translational product/s of the hybrid genes e.g. *E. coli*, an intermediate *in vitro* refolding step is introduced subsequent to the expression step.

The gene encoding for SK was first cloned in a bacterial plasmid in *E. coli* after isolation from the wild-type *S. equisimilis* genomic DNA according to the procedures disclosed in Indian patent application No. DEL/94) and in recent research publications available in the public domain (Pratap, J., Kaur, J., Rajamohan, G., Singh, D., and Dixit, K.L.,1996, *Biochem. Biophys. Res. Commun.* 227:303). Briefly, the complete structural gene of SK, including its promoter and upstream secretory signal sequence, was first cloned in the well-known cloning vehicle, plasmid pBR 322, by first constructing a genomic library of *S. equisimilis* H46A strain DNA (Accession No. MTCC 389) in the cosmid vector pHC 79, employing established procedures of rDNA research. The clones so obtained were then screened by the plasminogen-overlay method (Malke, H., Ferretti, J.J., 1984, *Proc. Nat'l. Acad. Sci.* 81:3557) to identify SK-producer clones, and the plasmid DNAs from such clones were then analyzed for the presence of heterologous, *S. equisimilis*-derived DNA (inserts). The inserts from a few positive clones were then digested with restriction enzymes Hind III and Pst I separately (to isolate smaller fragments carrying the SK gene), and the restriction fragments were ligated with pBR 322 at the respective R.E. sites. The transformants were again screened for SK-producer clones using the plasminogen-overlay method, and positive clones were isolated and DNA from these were analyzed with respect to insert size as for the cosmid clones previously. The plasmid carrying the smallest insert from the SK-secreting clones identified by the plasminogen overlay procedure was taken up for further genetic manipulation. This plasmid, designated pJKD-8, carried the SK gene under its own promoter and secretory signals (for map, refer to Fig. 2). The SK gene encoding for mature SK i.e. devoid of its 5'- regulatory and secretory sequences was then selectively amplified by PCR and cloned in a cloning vector; details of this procedure have been recently published (Pratap, J., Kaur, J., Rajamohan, G., Singh, D., and Dixit, K.L.,1996, *Biochem. Biophys. Res.. Commun.* 227:303). In the process of the present invention, the DNA corresponding to the ORF of SK has been modified with respect to its 5'-coding sequences so that after cloning in an expression vector under the control of a strong promoter, large

quantities of biologically active SK are produced intracellularly. The DNA sequence of the SK gene and the corresponding amino acid sequence of the mature protein are provided in Fig. 3. The restriction enzyme map of the SK gene is provided in Fig. 4.

The DNA sequences encoding for the fibrin binding domains of human fibronectin were selectively amplified from known plasmids containing cloned cDNA for the FN gene. Kornblihtt, A.R., Vibe-Pedersen, K., and Baralle, F.E.,1983, *Proc. Nat'l. Acad. Sci.* 80:3218. have cloned the cDNA encoding for the human fibronectin (FN) gene in a plasmid vector in *E. coli* (pFH1). This cDNA extends approx. 2.1 kb from the poly-A tail of the mRNA of fibronectin, around one-fourth of the estimated size of the human FN message (approx. 7900 nucleotides). By further mRNA "walking" type of experiments, these investigators carried out the construction of longer cDNA clones using synthetic oligonucleotides complementary the DNA of clone pFH1. By this method, cDNAs corresponding to the complete FN mRNA were prepared and cloned in several vectors (Kornhblitt, A.R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F.E., (1985) *EMBO J.* 4:1755). One such plasmid (pFH6) contained the entire sequences coding for the FBDs of the N-terminal region of human FN (see Fig. 5 for the map of this plasmid and Fig. 6 for the nucleotide and amino acid sequence of the FBD regions contained in this plasmid and Fig. 7 for its restriction enzyme map). Plasmid pFH6 served as the source for these sequences in the construction of the SK-FBD hybrids. The fibrin binding domains located in the N-terminal region of human FN gene were selectively amplified by PCR using specially designed oligonucleotide primers that hybridized with DNA sequences flanking the FBD DNA segments to be amplified. These primers also contained non-hybridizing sequences at their 5'-ends that provided the intergenic sequence (i.e. between the SK and FBD DNA) as well as a restriction site through which the amplified DNA could be ligated with the SK gene in-frame in a plasmid vector. The cloned hybrid gene was then expressed in *E. coli* so as to produce large quantities of the chimeric polypeptide. This protein was then isolated from the *E. coli* cells and subjected to a process of purification and refolding to a biologically active form. Similarly, different designs of the SK and FBD hybrids were then constructed using recombinant DNA methods, expressed, and isolated in biologically active, purified forms. Analysis of the properties of these proteins established that these indeed possessed the functional properties expected from their design i.e. plasminogen activation ability as well as fibrin selectivity. They also displayed the additional desired property of a very slow initial kinetics of PG activation, which, after a lag of varying between approximately 10-25 minutes depending on the construct, was overcome, leading to high rates of PG activation comparable to native SK.

The invention is illustrated by the following examples which, however, should not be read in a restrictive sense. These are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

I. General methods used in Examples

1. Recombinant DNA methods: In general, the methods and techniques of rDNA well known in the area of molecular biology were utilised. These are readily available from several standard texts and protocol manuals pertaining to this field of the art, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (IInd edition, Cold Spring Harbor Press, New York., 1989; McPherson, M. J., Quirke, P., and Taylor, G.R., [Ed.] PCR: A Practical Approach. IRL Press, Oxford., 1991). However, pertinent details in the context of specific experiments describing the present invention, particularly where modifications were introduced to established procedures, are indicated in the Examples wherever relevant.

2. **Casein-plasminogen overlay for detection of SK activity**: bacterial colonies producing strpetokinase can be routinely detected by overlay of casein and human plasminogen in soft agar (Malke, H., Ferretti, J.J., 1984, Proc. Natl. Aca. Sci. 81:3557). Ten ml soft agarose mixture consisting of 0.8 % agarose, 10 % skimmed milk, approx. 200 ug of human plasminogen, 150 mM NaCl, and 50 mM Tris-Cl (pH 8.0) is poured on top of the plates. The plates are incubated at 37 °C for 2-6 h. Positive streptokinase activity is indicated by the appearance of zones of clearance around the colonies (halo formation).

3. Zymography: proteins from total cell lysates are separated on 10 % SDS-PAGE. After completion of electrophoresis run, gel is washed with 2.5 % Triton X-100 to remove any SDS. It is then thoroughly rinsed with standad buffer 0.05 M Tris Cl (pH 7.5) for Triton X-100 removal. The gel is laid on 0.6 % agarose plate containing 10 % skimmed milk and 0.5 mg/ml human plasminogen. After incubating at 37 °C for 2-3 h, an active SK band is visualized as a clear band.

4. SDS-PAGE analysis of proteins: SDS-PAGE is carried out, essentially according to Laemmli, U.K., 1970, *Nature* 227:680 with minor modifications, if needed. Briefly, protein samples are prepared by mixing with an equal volume of the 2X sample buffer (0.1 M Tris Cl, pH 6.8; 6 % SDS; 30 % glycerol; 15 % beta-mercaptoethanol and 0.01% Bromophenol Blue dye). Prior to loading onto the gel, the samples are heated in a boiling water bath for 5 min. The discontinuous gel system usually has 5 % (acrylamide conc.) in the stacking and 10 % in the resolving gel. Electrophoresis is carried out using Laemmli buffer at a constant current of 15 mA first, till the samples enter the gel and then 30 mA till the completion. On completion of electrophoresis, gel is immersed in 0.1% coomasie Blue R250 in methanol: acetic acid: water (4:1:5) with gentle shaking and is then destained in destaining solution (20 % methanol and 10 % glacial acetic acid) till the background becomes clear.

6. Immuno-assay of Western blotted proteins: Western blotting of the proteins from *E. coli* carrying plasmid encloded intracellular streptokinase is carried as detailed (Towbin, H., Stachelin, T., Gordon, T., 1979, *Proc. Natl. Acad. Sci.* 76: 4350. The cultures are grown to 600 nm of 0.5-0.6, and are induced with 1-5 mM IPTG. The cells are centrifuged. The pellet is resuspended in cell lysis buffer and the supernatant obtained after high-speed centrifugation. These fractions are resolved on the 10 % SDS-

PAGE. The gel is equilibrated with the transfer cell buffer (25 mM Tris, 175 mM glycine in 20 % methanol) and is blotted electrophoretically on to the nitrocellulose membrane at 50 V for 3 h. The blot is blocked with 5 % skimmed milk (Difco) in PBS (Phosphate buffer saline) for 14-16 h at 4 °C. The blot is further washed with 0.1 % Tween-20 in PBS. The blot is incubated with the anti-SK antibodies (raised against pure *S. equisimilis* SK in rabbit) in 40 ml of PBS conatining 5 % skimmed milk for 3 h at room temperature with gentle shaking. The blot is washed with 0.1 % Tween 20 in PBS three times for 15 minutes each. Again it is blocked with PBS-skimmed milk for 15 min with gentle shaking at room temperature and further incubated with peroxidase-conjugated goat anti-rabbit immunoglobulins at a dilution of 1:5000 in 20 ml of PBS-skimmed milk (5 %) for two 2 h at room temperature with gentle shaking. The filter is again washed with 0.1% Tween 20 in PBS for three times (15 min each). The colour reaction for HRP-linked secondary antibodies is carried out by immersing the blot in 10 ml of reaction buffer solution having 10 mg of DAB (di-amino benzidine) and imidazole each. The reaction is terminated by washing with distilled water.

6. **Streptokinase assay using chromogenic peptide substrate:** plasminogen activator activity of streptokinase is assayed according to Jackson, K.W., Esmon, N., Tang, T., 1981, *Methods in Enzymology* 80: 387. One hundred ul of appropriately diluted streptokinase samples, 25 ul of sample buffer (0.15 M Tris-Cl buffer, pH 7.5) and 15 ul of human plasminogen solution (0.5 mg/ml in 0.05 M Tris-Cl, pH 7.5) are mixed together. The tubes are incubated at 37 °C for 15 min, after which18 ul of NaCl (1.77M in 0.0.32 M Tris-Cl, pH 7.5) is added. The amount of plasmin generated in the first stage is measured by further addition of 12 ul of plasmin-specific chromogenic substrate, Chromozyme-PL (Boehringer-Mannheim, Germany), 5 mg/ml in water, and the tubes are again incubated at 37 °C for 10 min. After this incubation, 0.4 ml acetic acid (0.2 M) is added to terminate the reaction. The release of yellow-colored 4-nitroaniline is monitored at 405 nm spectrophotometerically. Appropriate dilutions of *S. equisimilis* streptokinase obtained from WHO, Hertfordshire, U.K. is used as a reference standard for calibration of international units in the unknown preparation. Protein concentration is estimated according to the method of Bradford, M.M., 1976, *Anal. Biochem.* 72: 248) using BSA as a standard. Bradford's reagent (Biorad Inc., USA) is utilized according to the manufacturer's instructions. For estimaing the concentration, protein-samples in phosphate buffer are made to 800 ul. To this, 200 ul Bradfor's reagent is added and is mixed thoroughly. The reaction is maintained at room temperature for 5 min and absorbance at 595 nm is monitored. The specific activity for PG activation (I.U./mg protein) of an unknown preparation of SK or SK-PBD is thus determined from the SK assay and protein estimation data.

7. **Fibrin clot assay for SK:** This test is performed to determine the clot lysis ability of any thrombolytic drug, such as streptokinase, urokinase or tissue plasminogen activator, and is adapted from British Pharmacopia (1980 edition).

Reagents : (i) 100 mM citrate phosphate buffer, pH 7.1 containing 0.8 % BSA (referred to as buffer-1). (ii) Bovine fibrinogen (Cohn Fraction-I, obtained from Sigma Chemical Co., St. Louis, USA), 2.5 mg/ml prepared in buffer -1). (iii) Bovine thrombin (obtained from Sigma as a lyophilized powder). Stock solution of SK, 500 I.U./ml, prepared in sterile water and stored in aliquots of 50 ul each at -70

°C. Before use, one aliquot is thawed and diluted to 50 I.U./ml in buffer-1. (iv) Human plasminogen (Boehringer Mannheim, Germany) stock 1mg/ml, prepared in sterile water. Stored in aliquots of 100 ul each at -70 °C. (v) Standard SK (from W.H.O., obtained from Dr. P.J. Gaffney, Division of Haematology, N.I.B.S.C., Blanche Lane, Potters Bar, Hertfordshire, EN 6 3QG, U.K.). The standard SK vial is composed of 700 international units of SK (in lyophilized form alongwith stabilizers). A complete vial should be dissolved in 700 ul of sterile dist. water to obtain a concentration of 1000 I.U./ml. The dissolution should be carried out either at 4 °C or by keeping all the solutions on ice. The dissolved SK is then aliquoted into convenient sizes and stored at -70 °C. Prior to carrying out clot lysis assay an aliquot of 1000 I.U./ml (stock) is thawed and diluted further in cold buffer-1 on ice. Dilutions (A to D, below) are prepared serially in the following way using a new pipette tip for each transfer.

A. 10 ul of stock + 990 ul of buffer-1 = 10 I.U./ml.
B. 500 ul of A + 500 ul of buffer-1 = 5 I.U./ml.
C. 500 ul of B + 500 ul of buffer-1 = 2.5 I.U./ml.
D. 500 ul of C + 500 ul of buffer-1 = 1.25 I.U./ml.
(All dilutions are tempered at 37 °C prior to use in the clot test as are the other solutions to be used.)

Two hundred ul of each dilution is used in the clot lysis reaction mixture. One unit of SK (present in 200 ul of SK dilution B) is just sufficient to lyse a standard fibrin clot in approximately 5 min at 37 °C.
Clot lysis test protocol: (a) Preparation of clot (negative control): During each step, the contents of the tube are gently mixed.

Step 1 : add 450 ul of buffer -1 to a small glass tube (0.8 mm internal diameter).

Step 2 : add 50 ul of bovine thrombin (50 I.U./ml) solution to the tube.

Step 3 : add 100 ul of 1 mg/ml plasminogen to the solution in tube.

Step 4 : add 400 ul of 2.5 mg/ml fibrinogen to the solution in tube.

Immediately after step 4, the tube is kept at 37 °C in a water bath without shaking. A standard clot forms within 30 -40 seconds. The I.U./ml in the unknown is determined in a similar manner after appropriate dilution.

B) Clot lysis with thrombolytic agent (SK): When clot lysis is to be performed using standard SK, all the steps i.e. 1, 2 and 3 are carried out as described above, except that at step 1, only 250 ul. of buffer-1 is added. Also, at step 4, 200 ul from the appropriate dilution of SK containing 1-2 units (as described under Reagents, above) is premixed with 400 ul of fibrinogen solution in a separate eppendorf tube, and rapidly equilibrated to 37 °C in water bath. This mixture is then added to the clotting reaction at step 4, described above. The tube is then incubated as previously. A clot is formed in the same or lesser time as above, but is now followed by its lysis. The time for complete lysis is noted down using a stop watch. The time for lysis depends upon the amount of SK used in the mixture. Lysis time by a particular unit of standard SK (i.e. lysis time of 5 min by 1 I.U. of SK) is

taken as a standard. The unknown preparation of SK should be diluted appropriately to obtain a lysis time of approximately 5 min, which can then be used to calculate the units of SK present in that unknown preparation.

8. Kinetic assays for determining the HPG activation by SK or SK-FBD chimeras: A one-stage assay method (Shi, G.Y., Chang, B.I., Chen, S.M., Wu, D.H. and Wu, H.L., 1994, *Biochem. J.* 304:235. Wu, H.L., Shi, G.Y., and Bender, M.L., 1987, *Proc. Natl. Acad. Sci.* 84: 8292. Wohl, R.C., Summaria, L., and Robbins, K.C., 1980, *J. Biol. Chem.* 255:2005) was used to measure the activation of HPG by SK or SK-FBDs. Varying concentrations of either SK or SK-FBD chimeric protein (10 nM-200 nM) were added to 100 ul-volume micro-cuvette containing 1 uM of HPG in assay buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM chromogenic substrate and 0.1 M NaCl). The protein aliquots were added after addition of all other components into the cuvette and bringing the spectrophotometric absorbance to zero. The change in absorbance at 405 nm was then measured as a function of time in a Shimadzu UV-160 model spectrophotometer.

9. Assay for determining the steady-state kinetic constants for HPG activator activity of SK and SK-FBD constructs: To determine the kinetic parameters for HPG activation, fixed amounts of SK or SK-FBD(4-5), 1 nM, were added to the assay buffer containing various concentrations of HPG (ranging from 0.035 to 2.0 uM) in the 100 uL assay cuvette as desribed above. The change in absorbance was then measured spectrophotometrically at 405 nm for a period of 30-40 min at 22 °C. The kinetic parameters for HPG activation were then calculated from inverse, Michaelis-Menton, plots by standard methods (Wohl, R.C., Summaria, L., and Robbins, K.C., 1980, *J. Biol. Chem.* 255:2005).

10. Radioactive fibrin clot preparation: 50 uL ul of (2.5 mg/ml) cold fibrinogen was mixed with 50 ul (9 $X10^5$ cpm) of ^{125}I- labelled fibrinogen (specific activity 8 $X\ 10^5$ cpm/ug of fibrinogen) and added to the mixture of 1.1 uM HPG and 0.25 units of human/bovine thrombin in 0.1 M citrate phosphate buffer, pH 7.5 containing 0.8 percent BSA in a total volume of 1 ml in a glass tube (1.3 X 12 cm). The clot was formed by incubating the mixture at 37 °C for 2 min. The clot was then washed thrice with 2 ml of TNT buffer (50 mM Tris-Cl buffer, pH 7.5, containing 38 mM NaCl and 0.01 percent Tween-80) for 2 min at 37 °C. As required the non-radioactive fibrin clots were prepared by omitting ^{125}I-labelled fibrinogen from the clotting mixture.

11. Clot lysis in the presence of human plasma: ^{125}I-fibrin clot lysis was carried out in the presence of 2 ml citrated human plasma containing different concentrations of either SK or SK-FN (ranging from 100 to 200 nM) at 37 °C. The reaction tubes were rotated slowly at 37 °C and 0.1 ml aliquots were removed at regular intervals to measure the soluble ^{125}I-fibrin degradation products by measuring the amount of radioactivity released using a gamma counter. The total radioactivity of each clot was determined by measuring the radioactivity of the respective tube before taking out any aliquots.

12. Clot lysis in the presence of human fibrinogen: ^{125}I-fibrin clot lysis was also carried out in the presence of various concentrations of human fibrinogen (ranging from 1 to 4 mg/ml) containing 100 nM

of either SK or SK-FN. Clot lysis was also performed in the presence of fixed fibrinogen concentration (2 mg/ml) and different concentrations of SK or SK-FBD protein construct (ranging from 50 to 200 nM). The reactions were incubated at 37 °C with gentle shaking and the release of ^{125}I-fibrin degradation products as a function of time was measured as described previously.

Brief Description of the Drawings

Fig. 1. Schematic representation of different chimeric proteins prepared by the fusion of SK and FBDs.

Fig. 2. Map of plasmid pJKD-8, containing SK gene from *S. equisimilis* H46A..

Fig. 3. DNA and protein sequence of streptokinase of *S. equisimilis* H46A (GeneBank accession number: gb/K02986/STRSKC).

Fig. 4. Partial restriction enzyme map of DNA encoding for SK.

Fig. 5. Map of plasmid pFH-6, containing FBD 1 to 5 encoding sequences according to Kornhblitt, A.R., Umezawa, K., Vibe-Pedersen, K. and Baralle, F.E., (1985) *EMBO J.* 4:1755.

Fig. 6. DNA and protein sequence of the gene-segment encoding for FBDs 1-5 of human fibronectin (the DNA sequence has been obtained from EMBL; the file and accession no.'s are ID-HSFIBI and X 02761,K 00799, K 02273,X 00307, X 00739).

Fig. 7. Restriction enzyme map of DNA encoding the five N-terminally located FBDs of human fibronectin.

Fig. 8. Map of pET23(d).

Fig. 9. Flow chart schematically depicting the main steps in the construction of a plasmid vector for the expression of the native SK gene of *S. equisimilis* H46A.

Fig. 10. Flow diagram schematically depicting the main steps involved in the repair of the vector pET23(d)SK i.e construction of expression vector pET23(d)SK-NTRN (NTRN: abbrev. form for N-terminally repaired with native sequence).

Fig. 11. Nucleotide sequence of SK-NTRN gene.

Fig. 12. Predicted secondary structure of native (A) and translationally silently modified (B) 5'-ends of the SK gene sequence.

Fig. 13. Schematic flow diagram depicting the main steps in the construction of a plasmid vector [pET23(d)SK-NTR] for the intracellular hyper-expression of a semi-synthetic SK gene in which the 5'-end of the open-reading-frame for SK was selectively modified in a translationally silent manner at the DNA level, so that it encoded for the primary structure of *S. equisimilis* SK.

Fig. 14. Nucleotide sequence of SK-NTR gene.

Fig. 15. Schematic depiction of the intergenic region of the chimeric SK-FBD(4,5) gene highlighting the design of a gly-gly-gly sequence, a transglutaminase cross-linking site and several unique restriction enzyme sites wherein different inter-genic cassettes can be conveniently swapped into this region. Also shown is the location of the natural Bsm I site in the SK gene which was exploited as the common junction point for joining the FBD sequences to the SK gene.

Fig. 16. Flow diagram depicting the main steps in the construction of plasmid pSKMG400, containing the SK-FBD(4,5) hybrid DNA block composed of FBD(4,5) sequences linked to the intergenic sequences at its 5'-end, and the SK gene fused in-frame at the 3' end.

Fig. 17a. Scheme depicting the cloning of the hybrid SK-FBD(4,5) cassette into pET23(d)SK-NTR for intracellular expression of SK-FBD(4,5) chimeric protein in *E. coli*.

Fig. 17b. DNA sequencing data of SK-FBD(4,5) hybrid cassette in T7 expression vector, pET23(d).

Fig. 18. Schematic flow diagram for cloning of SK-FBD(1,2) hybrid gene in pBluescript, to obtain pSKMG400-FBD(1,2)].

Fig. 19a. Schematic description of steps involved in the cloning of the hybrid gene-construct SK-FBD(1,2) into expression vector pET23(d)SK-NTR for intracellular expression of SK-FBD(1,2) chimera in *E. coli*.

Fig.19b. DNA sequencing data of SK-FBD(1,2) hybrid cassette in T7 expression vector.

Fig. 20. Scheme of steps involved in the construction of hybrid gene block composed of DNA encoding for FBD(4,5) and residues 1-63 of SK by the Overlap Extension PCR technique.

Fig. 21a. Scheme depicting steps involved in the cloning of the FBD(4,5)-SK gene block for expression of FBD(4,5)-SK chimera in *E. coli*.

Fig. 21b. DNA sequencing data of FBD(4,5)-SK gene block as present in the T7 expression vector pET23(d)-FBD(4,5)-SK.

Fig. 22 a. Flow chart depicting schematically the steps involved in the construction of FBD(4,5)-SK-FBD(4,5) hybrid gene in pET23(d) expression vector.

Fig. 22 b. DNA sequencing data of FBD(4,5)-SK-FBD(4,5) gene block as present in the T7 expression vector pET23(d)FBD(4,5)-SK-FBD(4,5).

Fig. 23. Purification of SK-FBD(4,5) protein expressed in *E. coli* by a one-step affinity chromatographic procedure.

Fig. 24. Clot lysis by purified SK-FBD(4,5) chimeric protein in a plasma milieau.

Examples

Example 1. High level intracellular expression of biologically active native and N-terminally truncated Streptokinase in *E. coli*.

In order to express native, full-length *S. equisimilis* strain H46A Streptokinase intracellularly in *E. coli* the SK gene was transferred from the plasmid vector construct pJKD-55 by digesting with Nco I and Sal I restriction enzymes (R.E.) which liberated the SK open-reading-frame (ORF). The construction of pJKD-55 has been described in detail in Indian Patent application No. DEL/94 dated 30.12.94, which also describes the isolation of the SK gene and preparation of its modified analogs from its host, *S. equisimilis* H46A, cloning and expression in heterologous hosts such as *E. coli*, and the purification and biological properties of the expressed polypeptides.

The DNA segment liberated from pJKD-55 was then cloned into pET-23(d) (see Fig. 8 for map of this plasmid) which had also been treated with the same enzymes (Nco I and Sal I) to obtain cohesive ends compatible with those of the SK gene (see Fig. 9 for the scheme used for this

purpose). This vector contained an initiation codon in-frame with the Nco I site of pET-23(d). Upon ligation, the SK open-reading-frame could be recreated, but one modified at the N-terminal end, together with an additional ATG at the 5' end emanating from the re-formed Nco I site.

The construction of pET23(d)-SK was carried out as follows. Approx. 3 ug each of pET23(d) and pJKD-55 plasmid DNAs were digested (separately) with 20 units each of Sal I (37 C for 6 h), followed by 15 units each of Nco I in 20 ul reactions at 37 °C for 10 h. After stopping the reactions by heat treatment (65 °C, 10 min), followed by phenol-chloroform extraction and ethanol precipitation of the DNA, the digests were run electrophoretically on a 1.2 % agarose gel to isolate the needed DNA fragments i.e. insert, carrying the SK gene from pJKD-55, and the linearized vector pET23(d) [see Fig. 9]. The respective fragments were purified from the gels using the Prep-A-Gene DNA purification kit of BioRad Inc., CA, USA. The insert and double-digested, linearized vector DNAs were then ligated at an approx. 3:1 molar ratio (~350 ng of vector and 400 ng of the insert liberated from pJKD-55) in a 20-uL reaction using standard ligation conditions at 16 °C for 12 h. After this duration, the ligase was heat inactivated (60 C, 15 min) and one-fifth of the ligation reaction was directly used to transform *E. coli* XL-Blue electrocompetent cells using the following electroporation conditions with 2 mm internal diameter electroporation cuvettes (obtained from BioRad Inc., Richmond, CA, USA): voltage, 2.5 KV; resistance, 200 ohms, and capacitance 25 uF. Six transformants were picked up from Amp-LB plates on which the transformed cells were plated at various dilutions. Individual colonies were inoculated into 10 ml LB-Amp media to prepare plasmid DNA by standard methods. The isolated plasmids were then screened electrophoretically on the basis of molecular size to identify the positive clone/s. All six clones were positive by this criterion. In order to express full-length SK containing all of the amino acid residues of mature *S. equisimilis* SK (Fig. 3), the native N-terminal was repaired using a synthetic "cassette" approach (refer to Fig. 10 for the scheme followed for the repair of the SK gene). The portion of the repaired SK gene at the 5' end in pET23(d)SK was obtained through PCR using the primers RG-6 and RG-7 with the following sequence and target specificity.

RG-7 (forward primer)

5'-ATT GCT GGA CCT GAG TGG CT-3'
(specific for the first seven codons of the SK gene; Cf Fig. 11)

RG-6 (reverse primer)

5'-TGG TTT TGA TTT TGG ACT-3'
(specific for codons 57-62 of SK gene)

The PCR was carried out using as the template, plasmid pSKMG-400, which contained the full-length native SK gene. This plasmid was constructed by cloning the Nco I-Sal I fragment obtained from pJKD-55 followed by T4 DNA polymerase-catalyzed fill-in of the two ends (to

obtain blunt ends) and cloning at the Eco RV site of plasmid BlueScript KS⁻ (Stratagene Inc., WI, USA). The two PCR primers were designed to amplify the N-terminal portion of the native SK gene upto a unique restriction site in the gene which could be utilized for recloning the amplified PCR product back into pET23(d)-SK for expression of protein. Moreover, the 5' end of the RG-7 primer started with ATT, coding for Ile, the first residue of the mature SK gene, so that the PCR-amplified SK gene segment could dock in-frame with the nucleotides ATG, formed at the NcoI-cut and refilled end of the expression vector, thus juxtaposing the initiation codon in-frame for the repaired SK ORF. The following PCR conditions were used for the amplification reaction (100 uL total): approx. 10 ng pSKMG-400 as template, 20 pmol each of the RG-6 and RG-7 primers, 1 ul (2.5 units) of pfu DNA polymerase (Stratagene Inc.), 200 uM of each dNTP's, 10 uL of the standard buffer (10 X conc. provided by the Stratagene Inc.). The following cycling parameters were used: 'hot start' for 5 minutes at 92 °C, denaturation at 92 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. A total number of 30 cycles, and a final extension of 10 min at 72 °C for allowing the completion of any of the incomplete amplified products, were provided. The PCR showed a single band of 160 bp as evidenced by electrophoresis on a 1.2 % agarose gel. For cloning the PCR product into pET23(d)-SK vector, approx. 10 ug pET23(d)-SK vector was digested with 25 units of NcoI restriction enzyme in a 100 ul reaction using the buffer (NEB-4) supplied by New England Biolabs, Inc., and by incubating at 37 °C for 6 h. The completion of NcoI digestion was checked by loading 5 ul of the reaction mixture on a 0.7% agarose gel. After confirming the digestion, the NcoI site was filled-in (i.e. made blunt ended) using T4 DNA polymerase in the presence of all four dNTP's in a 85-ul reaction as follows. Seven five ul of above-mentioned Nco I digestion mixture was supplemented with 4 ul DTT (100 mM stock), 4 ul dNTP's from a dNTP stock (2 mM), and 2 ul (6 units) of T4 DNA polymerase. The reaction was incubated at 37 °C for 1 h after which it was stopped by adding EDTA (10 mM final conc.) and heating at 75 °C for 10 min. The DNA was then ethanol-precipitated. The precipitated DNA was dissolved in 40 ul TE and was digested with Afl II restriction enzyme in a 60-ul reaction at appropriate reaction conditions as recommended by the supplier. Separately, 40 ul of the PCR-amplified DNA reaction, prepared using pSKMG-400 vector as substrate to supplement the deleted portion of the SK gene, was also digested with Afl II restriction enzyme, followed by running on low melting agarose gel (1 %) to separate the vector and insert DNA pieces [the insert contained a blunt end, and an Afl II-site compatible cohesive terminus at the other end, thus making it suitable for facile ligation with the vector, which had been similarly treated with Nco I, followed by a fill-in reaction with T4 DNA polymerase to obtain a blunt end, followed by a digestion with Afl II]. The required pieces of DNA were isolated from the electrophoresis gels as small agar blocks after visualization under transilluminated UV radiation, and were purified from the agarose by beta-agarase enzyme. One unit of beta-agarase per 100 ul of agarose gel approximately in the 1X beta-agarose buffer (New England Biolabs Inc.) was employed to digest the agarose and to purify the DNA according to the protocol recommended by the supplier (New England Biolabs Inc., USA). The purified DNAs were quantitated and vector and insert were ligated in a 1:5 molar ratio in a 20 ul reaction, carried out at 16 °C for approx. 18 h. For the ligation reaction, 2 ul of 10 x ligase buffer, 1ul of 10 mM rATP stock, and 2000 Weiss units of T4

DNA ligase were used. The DNA from the ligation mixture was precipitated with n-butanol, and used directly to transform electrocompetent *E. coli* JM 109 cells. The transformation mixture was plated on LB-Amp plates. The positive clones (repaired pET23(d)-SK) were screened from the wild-type background on the basis of Nco I digestion (the insertion of the PCR amplified segment in the vector would result in the loss of the Nco I site). Two of the clones (pETSK-NTRN, for 'N-terminally repaired, native') obtained after this screening were further confirmed using Sanger's method of nucleotide sequencing, which showed complete fidelity with the full-length native sequence of the *S. equisimilis* SK gene (Cf. Fig. 11) except the presence of an extra ATG codon at the 5'-end of the ORF, and no mutation/alteration at the upstream promoter regions or downstream sequences in the plasmid could be observed. DNA from these confirmed clones were then transformed into *E. coli* BL-21 strain, and expression of intracellular SK in liquid culture was examined after induction with IPTG according to the protocol described earlier, essentially by analyses of cell-lysates on SDS-PAGE. However, no band corresponding to standard SK was visible on SDS-PAGE. The possibility of the presence of low levels of SK was then checked by Western Blotting analysis of the lysates as it is a more sensitive method when compared to a direct examination of the SDS-PAGE gels by Coomassie staining. In this case, indeed, a faint band corresponding to the position of standard SK on the Western blots could be clearly discerned, which showed that the levels of expression of the native SK gene in was poor.

The possibility that the sequences in the native SK gene corresponding to the N-terminal residues could be forming strong secondary structure/s in the gene transcripts that might be hindering the expression was examined through computer-assisted analysis using the program DNASIS (version 5.0). This unambiguously demonstrated that the potential for forming highly stable secondary structure by the N-terminal end of the SK gene was appreciably strong (free energy approximately -10 Kcal/mol; see Fig. 12A). Translationally silent mutagenesis of the gene at its 5' end was then carried out to disrupt and/or reduce this secondary structure by replacement of GC rich codons (that are more likely to promote secondary structure-formation in the mRNA transcript) with AT-rich codons, wherever possible. Through this procedure several sequence/s, altered specifically at the 5'-end and possessing lowered stability (-6 to -5 Kcal/mol) as compared to that of the native sequence, were obtained. One of these sequences, that resulted in maximal destabilisation of structure-forming potential, to approx. -5 Kcal/mole (Fig. 12B), was chosen for the expression studies.

The preparation of an expression vector containing full-length SK gene encoding native SK but with a modified (non-native) DNA sequence at its 5'-end with less structure-forming potential was carried out as shown schematically in Fig. 13 using a combination of synthetic DNA cassette incorporation and PCR-based strategy. The alternate sequence to be incorporated at 5'-end of the SK gene was provided through two homologous synthetic oligonucleotides (SC-I and SC-II, complementary to each other excpt for overhangs at the end), whose sequence is shown below. Also indicated in bold type are the altered nucleotides which resulted in a lowering of structure forming potential in the 5'-end of the SK gene.

SC-I

5'-C ATG ATA GCT CGT CCT GAA TGG CTA CTA GAT CGT CCT TCT GTA AAT AAC AGC C-3'
(Partial NcoI site)

SC-II

5'-AA TTG CCT GTT ATT TAC AGA AGG ACG ATC TAG TAG CCA TTC AGG ACC AGC TAT-3'
(Partial Mfe I site)

These carried two new restriction sites (NcoI and MfeI), introduced by silent mutagenesis using the computer program GMAP (Cf. Raghava and Sahni, 1994., BioTechniques vol. 16, pp. 1116-1123) without altering amino acid sequence encoded by this segment of DNA so as to facilitate the cloning of the repaired SK gene into the expression vector, pET23(d)-SK (see Fig. 13 for the overall cloning scheme for reconstruction of the N-terminal region of *S. equisimilis* SK gene). The alterations were carried out in two stages, as depicted schematically in Fig. 14. In Stage I, a translationally silent restriction site (Mfe I) was engineered close to the N-terminal end of the SK gene (overlapping codon numbers 17 and18 in the native SK sequence; see Fig. 3) since no unique site close to the N-terminal end was available for incorporating a synthetic DNA piece for purposes of altering this region in the plasmid. An upstream PCR primer (termed 'Mfe I primer') incorporating this potential Mfe I site (underlined in the sequence of the primer) was synthesized with the following sequence.

Mfe I primer: 5'-C-AGC-CAA-TTG-GTT-GTT-AGC-GTT-GCT-3'

A synthetic oligonucleotide containing an Afl II site (termedRG-6, which has been described before) was used as the downstream primer.

These two primers were utilized for the amplification of the SK sequences encoding the N-terminal region using pfu DNA polymerase. The following reaction conditions and cycling parameters were used. Pfu polymerase buffer (Stratgene Inc.), 200 uM each of the dNTP's, MfeI and RG-6 primers: 20 pmol each, pET23(d)-SK vector as template (2 ng), Pfu polymerase 1 ul (2.5 units), total reaction volume 100 ul. A 'hot start' was given for 5 min at 95 °C, followed by denaturation for 1 min at 95 °C, annealing for 1 min at 45 °C, and extension for 1 min at 72 °C. A final extension at 72 °C for 10 min was also incorporated in the program. As expected from theoretical considerations, a 141-bp long SK region was amplified. The PCR product was phenol-chloroform purified and precipitated using isopropanol after adjusting the salt concentration to 0.3 M with 3 M NaOAC. The precipitated product was dissolved in 25 ul sterile dist. water and kinased in a 30-ul reaction, after adding 3 ul Multicore buffer (Promega Inc., WI, USA), 1ul (10 units) of T4 PNK (Promega) and 1ul rATP (10 mM stock). The reaction mixture was incubated

at 37 °C for 2 h and then stopped by heat-inactivating at 65 °C for 20 min, and the DNA purified using phenol-chloroform and precipitated with 2 volumes of isopropanol. The pBluescript II KS(-) vector was digested with EcoRV restriction enzyme and then dephosphorylated using CIAP using a standard protocol. Both the kinased PCR product and dephorphorylated pBluescript II vector were quantitated by A260 measurements in a 100-uL cuvette, and the vector and insert DNAs were ligated in 1:10 molar ratio of vector: insert by taking 590 ng vector and 280 ng insert in a 20 ul reaction after adding appropriate amount of ligase enzyme (approx. 500 Weiss units) and ligase buffer containing rATP. The ligation reaction was incubated at 16 °C overnight. The ligation mixture was heat inactivated (65 °C, 30 min), the DNA was butanol-precipitated, and approx. one-fifth electroporated into *E. coli* XL-Blue electrocompetent cells. The transformants were screened by plating them on LB-Amp plates. Ten transformants were picked up and inoculated for minipreparation of plasmid DNA. The minipreps were then digested with MfeI and AflII enzymes sequentially to identify the positive clones containing the 141 bp insert. Unmodified pBluescript was kept as control. All the transformants were found to be positive by this criterion. This construct was labelled as p(MfeI-AflII)-SK.

Stage-II: The oligos SC-I and SC-II in equimolar amounts (approx. 270 ng each) in 25 uL were annealed by cooling their mixture from 80 °C to room temperature slowly. Approximately 5 ug of pET 23(d)SK and 10 ug p(MfeI-AflII)-SK vectors were digested with Afl II/Nco I and MfeI/AflII, respectively, for vector and insert preparations. Twenty five units each of MfeI and AflII were used for vector prepartion and 50 units were used for insert preparation. The enzymes were added in two shots of 12.5 units and 25 units in each of the reactions. The pET23(d)-SK vector was digested in a 60ul reaction, and p(MfeI-AflII)SK was digested in a 100-ul reaction. The pET23(d)-SK digestion mixture was run on a 1% low melting agarose gel for vector preparation and the p(MfeI-AflII) SK digestion mixture was run on a 2 % agarose gel for isolating the 115 bp insert. Both the vectors and insert bands were cut out from the agarose gel and were purified using beta-agarase and quantitated. Then, a mixture of Nco I and Afl II digested pET23(d)-SK vector, annealed SC-I and SC-II oligos, and Mfe I-Afl II insert of p(Mfe I-Afl II)-SK vector were ligated in a 3-piece ligation reaction in a 1:7:5 molar ratio in a 20 ul reaction (see Fig. 13). In the actual reaction, approx. 660 ng of the vector, 92 ng of the insert and 60 ng of the annealed oligos were taken. The mixture was ligated by adding 2000 Weiss units of ligase into the reaction. The reaction was incubated at 16 °C overnight. The ligation mixture was n-butanol-precipitated, dried, redissolved in 10 uL TE and approx. one-third used to transform *E. coli* XL-1 Blue electrocompetent cells. The transformants were screened on LB-Amp plates. Ten transformants were picked and inoculated for preparation of minipreps. All the minipreps alongwith pET23(d)-SK as control were digested with Nco I enzyme to search for the positive clones. Only one clone, as well as pET23(d)-SK, gave digestion with Nco I which indicated that the remaining 9 clones were positive for the desired construct. One of the clones was then completely sequenced by automated DNA sequencing using Sanger's dideoxy method, which showed that the N-terminal region was now full-length i.e. encoded the native SK sequence plus a N-terminal methionine, containing exactly the sequence expected on the basis of the designed primers, SC-I and SC-II with

the altered codons at the 5' end compatible with potential for secondary structure reduction in the mRNA transcripts(see Fig. 14). In addition, the DNA sequencing established that no other mutation was inadvertantly introduced in the SK ORF in during the reconstruction protocol. This vector-construct was termed as pET23(d) SK-NTR (N-terminally reconstructed). The plasmid DNA from this clone was then transformed into *E. coli* BL-21 DE3 strain for expression studies. The *E. coli* BL-21 cells were grown in liquid culture and induced with 2 mM IPTG at an OD_{600} of ~0.6 for the induction of SK, as detailed earlier. The cells were then pelleted by centrifugation and lysed in SDS-PAGE sample buffer and analysed electrophoretically by SDS-PAGE on 10 % acrylamide gel. It was observed that the level of SK (47 kD band) was approx. 25-30 percent of the total soluble proteins, a substantial increase compared to the very low expression observed in the case of the construct with the native N-terminus (pET 23(d)-SK-NTRN).

Example 2. Harvesting of intracellularly expressed SK from *E. coli*; purification of SK and characterisation of highly pure and biologically active SK.

Glycerol stocks of *E. coli* BL-21 strain harbouring plasmid pET23(d)SK-NTR, maintained at -70 C, were used to prepare a seed culture by inoculating freshly thawed glycerol stock (approx. 100 uL) into 100 ml of LB medium (in a 500 ml conical flask) containing 50 ug/ml of ampicillin. The flask was incubated at 37 °C with shaking on a rotary shaker at 200 r.p.m. for 16 h. This culture was used to seed four 2 L Erlenmeyer flasks each containing 500 ml of the same medium (LB-Amp) using 5 % (v/v) of inoculum. The flasks were incubated at 37 °C with shaking (200 r.p.m.) for a duration till the absorbance at 600 nm had reached 0.5-0.6 (~2 h after inoculation). At this time, IPTG was added to the cultures to a final conc. of 2 mM and incubation, as before, continued for a further 3 h. The cultures were then chilled on ice and processed for the next step immediately. The cells from the culture media were harvested by spinning them down by centrifugation at 6000 x g in a GS-3 rotor (Sorvall) for 30 min at 4 °C. The supernatants were discarded and the combined cell-pellets carefully resuspended by vortexing in 65 ml of lysis buffer containing a chaotropic agent for effecting release of the cellular contents. The composition of the cell lysis buffer was as follows (final concentrations are given): 6 M guanidine hydrochloride and 20 mM sodium phosphate buffer, pH 7.2.

The *E. coli* cell suspension was shaken gently on a rotary shaker at 4 °C for 1 h to effect complete cell lysis. The lysate was then subjected to centrifugation at 4 °C for 15 min at 9000 r.p.m. The clear supernatant (containing approx. 300 mg total protein as determined by the Bradford method) was then processed further, as follows (all subsequent steps were conducted at 4 C, and all buffers and other solutions used were also maintained at 4 °C). The supernatant was diluted 6-fold in which the conc. of Gdn.HCl was 1 M; simultaneously, aliquots of a stock solution (0.5 M) of sodium phosphate buffer, pH 7.2, and NaCl (stock conc. 5 M) were added to obtain 20 mM and 0.5 M with respect to sodium phosphate and NaCl, respectively, in the diluted cell lysate supernatant (final volume 200 ml). The mixture was gently swirled for a few minutes,

and then loaded onto a 100 ml bed volume (4 cm internal diameter) axial glass column for hydrophobic interaction chromatography (HIC) on phenyl-agarose-6 XL (Affinity Chromatography Ltd., Isle of Man, U.K.) coupled with an automated liquid chromatography work-station (model Biocad Sprint, Perseptive Biosystems, MA, USA) capable of continuous monitoring of effluents at two wavelengths simultaneously, and formation of predefined gradients for elution. The column was pre-equilibrated with 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 7.2 (running buffer) onto which the bacterial cell lysate was loaded at a flow rate of 85 ml/h. The flow-through was collected, and the column washed with running buffer (400 ml total) at the same flow rate, followed by the same volume of running buffer devoid of NaCl (washing steps). The SK was then eluted with dist. water (pH 7.0) at a slower flow rate (35 ml/h). All the effluents were collected in fractions (25 ml each) and the SK activity as well as protein content in each fraction was determined. Virtually all of the loaded SK activity was found to bind to the column, less than 5 % of the total activity being found in the flow-through and washings. Approximately 85-90 % of the loaded SK activity was recovered at the dist. water elution step. SDS-PAGE analysis showed the presence of a predominant band of 47 kD migrating alongwith native SK (purified from *S. equisimilis* H46A) run as standard. The SDS-PAGE as well as the activity analysis showed the SK to be 85-90 % pure at this stage when compared to the unpurified cell lysate. The SK in the dist. water elute was then made 20 mM in sodium phosphate, pH 7.2 (running buffer) and loaded at a flow rate of approximately 300 ml/h onto DEAE-Sepharase Fast Flow (Pharmacia, Uppsala, Sweden) packed in a 1.6 x 20 cm axial glass column pre-equilibrated with the running buffer. The column was then washed with 200 ml of the same buffer, following which it was developed with a NaCl gradient (0-0.6 M) in running buffer (pH 7.2). All eluates from the column were saved with an automated fraction collector. Ten-ml fractions were collected, and SK activity as well as protein was estimated in each. Aliquots from each fraction were also analysed by SDS-PAGE to examine the relative purity of the eluted protein. The flow-through and washings were essentially devoid of SK activity, but approximately 80-85 % of the loaded SK activity eluted at around 0.35 M NaCl in the gradient as a single symmetrical peak (containing a total of 42 mg protein). The specific activity of this protein was 1.1×10^5 I.U./mg. On SDS-PAGE, it showed a single band co-migrating with standard natural *S. equisimilis* SK. A densitometric analysis of the SDS-PAGE gels revealed that the background protein/s in the final purified SK represented less than 2 % of the total Coommassie stainable content. The overall SK recovery with the purification process was found to be approx. 65 percent.

The purified recombinant SK expressed in *E. coli* was characterized physico-chemically by several other criteria in order to compare it with natural SK. By the clot lysis procedure, it showed a specific activity of 105,000 IU/mg, under conditions where natural SK from S. equisimilis strain H46A was found to have a specific activity of 110,000 IU/mg protein. Upon reverse phase high performance liquid chromatography (RP-HPLC) on C-18 columns, both SK types were indistinguishable, showing the presence of a single symmetrical peak at the same position when eluted with a gradient of gradually increasing ACN concentration. By UV spectroscopy, the recombinant SK was found to be identical to the natural SK. The N-terminal amino acid sequence

of rSK was found to be identical with that of natural SK, except for the presence of an extra methionine residue at the N-terminus (the sequencing was carried out for 25 cycles).

Example 3. Construction of a hybrid gene between SK and fibrin binding domains 4 and 5 of human fibronectin, its expression in *E. coli*, oxidative refolding, and purification of biologically active chimeric protein.

The scheme followed for the construction and expression of a chimeric (hybrid) gene between the DNA encoding for residues 1 to 383 of SK followed in-frame by the DNA coding for the FBD 4 and 5 of human fibronectin is shown in Fig. 16. A short linker segment coding for 3 glycine residues, in tandem, between the two gene-segments was incorporated into the design (termed 'intergenic sequence') (see Fig. 15) so as to provide flexibility to the expressed chimeric polypeptide product. In addition, a new terminator codon was introduced at the end of the FBD(4,5) DNA so that the hybrid ORF encoded for a polypeptide ending after the two FBDs. Thus, the design essentially had the following configuration: SK[residues 1-383]-(gly-gly-gly)-[FBD(4,5)]. In addition, a transglutaminase recognition site was also engineered in the gene-design directly after the intergenic sequence so that the expressed, hybrid protein could become covalently cross-linked to the fibrin strands of the clot (Fig. 15).

A two-stage PCR-based experimental strategy (Fig. 16) was employed to construct the hybrid gene. A gene-block containing the sequence coding for domains 4 and 5 was first selectively amplified using the plasmid pFHMG-60 as template. The latter contained the DNA encoding for all five human FBDs (Fig.16). This amplification reaction (PCR-I) was carried out with specifically designed forward and reverse primers with the following sequences.

Forward primer (MY 13):

5'-CCG **GAA TTC GCG CAA CAG ATT GTA** CCC ATA GCT GAG AAG TGT TTT CA-3'
Eco RI — Transglutaminase-recognition sequence — hybridizes to upstream FBD(4,5) sequence

Reverse primer (MY 14):

5'-GGC **CTT AAG AGC GCT CTA** ACG AAC ATC GGT GAA GGG GCG TCT A-3'
'clamp' — Afl II — Eco 47 III — stop codon — hybridizes to downstream FBD(4,5) sequences

Note:- In the above primer sequences, the 5'- non-hybridising sequences (bold) as well as the hybridizing ones, towards the 3'-ends of the primers that are complementary to selected segments of the human fibronectin gene's FBD(4,5)-encoding DNA sequences are shown. In the 5'-non-hybridizing ends were also incorporated new RE sites by 'silent' mutagenesis, a transglutaminase-encoding site and/or stop codon sequences, as indicated above (underlined). The start of the hybridizing sequences in MY-13 correspond to the begining of the sense strand sequence of FBD(4,5), namely residue 150 onwards (refer to Fig. 6 for the amino acid and DNA sequences of the fibrin binding domains of human fibronectin). In case of primer MY-14, the begining of the hybridizing sequence (antisense) correspond exactly to the codon for residue 259

of human fibronectin (Cf. Fig. 6). The 'clamp' mentioned in the figure refers to the extra nucleotides added at the 5' end of a primer to facilitate the digestion at the nearby R.E. site which, otherwise, is poorly digested when present at or near the end of a DNA fragment generated by PCR.

As described above, the forward primer contained a segment at its 3' end that was homologous with the 5' end of the DNA encoding for the FBD(4,5) sequences, and also contained a 5' (nonhybridizing) segment that encoded for a TG-recognition site as well as RE sites to facilitate the cloning of the PCR product in a plasmid vector. This plasmid, containing the FBD(4,5) gene-block and additional 5' sequences was then employed as template for a second PCR (PCR-II) using a set of primers (RG-3 and MY-14). Primer RG-3 was designed so as to contain the other desired elements of the intergenic segment viz., the codons for the gly-gly-gly residues, as well as those encoding for a small segment of SK (see Fig. 15 and 16) directly after a unique R.E. site (Bsm I) present naturally in the C-terminal region of the native SK ORF (approximately overlapping the codons 377 and 378 of the *S. equisimilis* SK open-reading-frame; Cf. Fig. 3 and4). This site was chosen as the common, junction-point between the two genes to be integrated. Additionally, unique restriction sites flanking the intergenic (i.e. in and around the -gly-gly-gly-) sequences were also designed into the upstream primer through translationally silent mutagenesis that could be exploited to substitute alternate oligonucleotide cassettes at the intergenic region of the hybrid gene that would provide altered flexibility and/or rigidity characteristics in the expressed polypeptide different from that provided by the $(gly)_3$ linker (Cf. Fig. 15).

The sequence of primer RG-3 is given below highlighting features incorporated in its design (bold letters denote non-hybridizing segments towards the 5'-end of the primer to distinguish these from the sequence complementary with respect to template DNA).

5'- G AAT GCT AGC TAC CAT TTA GCT GGT GGT GGC CAG GCC CAA CAG ATT GTA CCC-3'

Bsm I / Bst XI	Xcm I / Bal I	
(hybridizes to SK gene at codons 376- 383)	(-gly-gly-gly-)	segment hybridizing with the 5'-end of DNA block from PCR-I at the TG recognition site

The amplified DNA obtained from PCR-II using primers RG-3 and MY-14 was treated with Bsm I to "dock" it at the Bsm I site in the SK ORF in vector pSKMG400 at one end, and with Eco 47 III (which produces blunt-ends) to facilitate blunt-end cloning at the filled-in Bam HI site present after the SK ORF in the plasmid vector (Fig. 16).

The following reaction conditions and PCR parameters were used. PCR-I (final reaction conditions in a 100-uL reaction): 20 pmol of each of the MY-13 and MY-14 primers, pFHMG-60 vector as template (1 ng), 200 uM each of the dNTP's, Pfu polymerase 1ul (2.5 units). Pfu polymerase was added at 94 "C i.e. a hot start for 5 min was given to avoid non-specific

amplification. The following cycling conditions were employed: denaturation at 92 °C for 45 seconds, followed by annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A total number of 30 cycles were given, followed by a final extension for 10 min at 72 °C. The reaction yielded a single 360 bp PCR product as seen on a 2% agarose gel alongwith standard PCR markers. The amplified product was then cloned into pBluescript II KS$^-$ at the EcoRI site (refer to Fig 16) which had been introduced in the PCR product as a 5'-overhang. The PCR reaction mixture was purified using standard methods, and then kinased with T4 PNK enzyme. The kinased PCR product was ligated to concatamarize the PCR product in order to internalize the EcoRI site to facilitate cloning of the DNA using the procedure of Jung et al. (Jung, V., Pestka, S.B., and Pestka, S., 1990, *Nucl. Acids Res.* 18:6156). The ligated DNA mixture was then digested with EcoRI followed by electrophoresis on a 2% agarose gel to check the efficiency of the ligation and digestion steps. The band representing EcoRI-digested PCR product was cut out from the gel and purified. Approximately 400 ng of the PCR product was ligated with 1ug of pBluescriptII (KS$^-$) pre-digested with EcoRI and then dephosporylated. Approximately 2 ul of the ligation mixture was directly used to transform *E. coli* DH 5-alpha electrocompetent cells. The transformants were selected on LB-Amp plates containing IPTG and X-gal using blue and white colony selection. Ten white transformants were picked and taken up for minipreparation of plasmid. The plasmid DNAs were digested with EcoRI enzyme, and the digests analysed on 1.5 % agarose gels. The transformants releasing 360-bp insert (the size of the PCR product) were taken as positive clones. Nine of the ten clones were found to be positive by this criterion. One of the above clones was then confirmed for the absence of any unexpected mutations by automated DNA sequencing by Sanger's procedure, and used as template for PCR-II employing RG-3 and MY-14 as primers so as to add additional sequences at the 5' end of amplified FBD sequences in the cloned PCR-I product. The PCR-I had resulted in the addition of a TG recognition site and a stop codon onto the original FBD(4,5) gene-block, whereas the stage-II PCR was carried out to effect the addition of the poly-glycine linker and overlapping SK sequences onto the FBD(4,5) gene-block. The following reaction conditions and cycling parameters were used for carrying out PCR-II. Each of the dNTP's : 200uM, PCR -I product cloned in pBluescript II (KS$^-$) as template (linearized) 260 ng, RG-3 and MY-14 as forward and reverse primers (100 pmol each), 5% DMSO (v/v), 1 ul Taq DNA polymerase (2.5 units) in a total reaction-volume of 100 ul. Cycling parameters were similar to that of PCR-I except that the annealing temperature was lowered to 58 °C. An aliquot (aprox. one-tenth) of the PCR -II was run on a 1.5% agarose gel to check for amplification. As the Taq polymerase does not produce blunt ended PCR products (unlike pfu polymerase) but ones with a single-base overhangs (reference), the PCR product was first filled-in, and then kinased. These two modifications were carried out in a single reaction at 22 °C for 30 min using 10 units each of the T4 DNA polymerase and T4 PNK (total volume 85 ul). In addition, 8 nmol dNTP's as well as 1 mmol rATP were added to the reaction (all indicated conconcentrations are final). The reaction was stopped by adding EDTA to 10 mM followed by heating the tube at 70 °C for 10 min. The filled and kinased PCR product was subjected to a phenol-chloroform treatment and precipitated with two volumes of ethanol in the presence of 0.3 M sodium acetate. The pellet was redissolved in 20 ul of dist. water. Approx. 15 ul of this DNA was ligated in order to concatemarize the PCR

product. For doing the ligation, 1X Universal Buffer (supplied by Stratagene Inc.), 1ul (of a 10 mM rATP stock) and 400 weiss units of T4 DNA ligase were added to a 25 ul reaction. The reaction was incubated at 16 °C overnight. The ligase was heat-inactivated at 65 °C for 10 min. The concatamerized PCR product was then first digested with Eco47 III (approx. 20 units) in a 25 ul reaction at 37 °C for 6 h and then the DNA was digested with BsmI enzyme at 65 °C for 6 h after adding ~20 units of BsmI enzyme in the same reaction. In parallel, the vector pSKMG-400 (approx. 4 ug), containing the SK gene, was digested with BamHI enzyme according to standard protocol and the digested DNA was filled-in using T4 DNA polymerase in the presence of 100uM dNTP's and 0.5mM DTT. The reaction was incubated at 37 °C for 1 h. The reaction was stopped by heating the tube at 75 °C for 10 min. Then the BamHI filled vector was digested with BsmI enzyme by incubating at 65 °C for 6 h. The vector was purified by a phenol-chloroform tretament followed by a chloroform-isoamyl extraction, followed by ethanol precipitation of the DNA. Then the Eco47III and BsmI double-digested PCR product and BamHI-digested and filled-in plus BsmI-digested pSKMG-400 vector were ligated (refer to Fig. 16) in a 20-ul reaction by incubating at 16 °C for 14 h, after which the ligase was inactivated by heating at 70 °C for 10 min and then the DNA was precipitated with n-butanol. It was then used to transform *E. coli* XL-Blue electrocompetent cells. The transformed colonies were then selected on LB-Amp plates. Ten transformants were picked and screened for the presence of the diagnostic test, namely the release of a 372-bp fragment after digestion with NotI and BsmI enzymes, in contrast to a 295-bp fragment from the control plasmid, pSKMG400 since the positive clones contained the additional FBD(4,5) segment. Eight clones from the ten selectred turned out to be positive by this criterion. The positive clones were designated as pSKMG400-FBD(4,5). One of these was subjected to DNA sequencing which confirmed the presence of the expected sequence at the 5'-end, and a complete absence of any other mutation in the rest of the gene-block.

For the expression of the SK-FBD(4,5) hybrid gene intracellularly in *E. coli*, the scheme shown in Fig. 17a was followed. The BsmI-NotI piece from pSKMG400-FBD4-5 (begining from the C-terminal portion of the SK gene and carring the intergenic linker region, the FBD(4,5) segments, and ending after the stop codon for this ORF at the Not I site originating from the MCS of the parent vector) was transferred into plasmid pET23(d)SK-NTR digested with the same restriction enzymes. The digested vector and the DNA segment from pSKMG400-FBD(4,5) (insert) were isolated from 1% agarose gels after beta-agarase digestion, as described earlier. The vector and insert DNA were then ligated by standard procedures using a vector: insert molar ratio of 1:5 (approximately 590 ng of the BsmI/NotI double-digested vector and 250 ng of the BsmI/NotI double-digested insert in a 20-ul reaction). DNA from the ligation reaction was butanol precipitated and directly used to transform *E. coli* XL-1 Blue electro-competent cells. Transformants were selected on LB-Amp plates. Ten transformants were picked up and taken up for plasmid minipreparation. The miniprep DNAs were digested with BsmI and NotI enzymes, alongwith pSKMG400-FBD(4,5) as control. Three clones gave BsmI/NotI insert equal in size to that of the insert liberated from pSKMG400-FBD(4,5).One of the clone was then fully sequenced by automated DNA sequencing in and around the SK insert (see Fig.17b for the gene sequence).

All the 3 positive clones were transformed into *E. coli* BL-21 strain and were induced for the expression of SK-FBD(4,5) hybrid protein using the standard protocol described before. The *E. coli* BL-21 cells harboring the plasmid pET-SK-FBD(4,5) were induced with 2 mM IPTG at ~0.6OD_{600} and were further incubated for 3 h at 37 °C. In parallel, cultures were also growm where IPTG addition was omitted (uninduced controls). Cells from 1.5 ml of the cultures were pelleted down by centrifugation and were directly lysed in 100 ul SDS-PAGE sample buffer. After high-speed centrifugation (8000 g x 20 min) to pellet undissolved components, approx. 25 ul of the supernatunt of each of the samples (alongwith lysate from pET23(d) SK-NTR, as positive control) was loaded onto 10 % SDS-PAGE gel and subjected to electrophoretic analysis. The gels showed distinct bands of 57 kD in the IPTG-induced cultures (roughly representing 20-25 % of the total Commassie-stained protein bands in the gel), indicating that the hybrid SK-FBD(4,5) fusion protein had been expressed at high levels. In the case of pET23(d)SK-NTR harboring cultures, a band corresponding to 47 kD, the position of native SK, was observed. In parallel, SDS-PAGE gels were subjected to the plasminogen-overlay procedure, which showed distinct zones of clearance by the 57 kD hybrid protein. however, these zones were produced with a distinctly slower rate in comparison to those produced by native SK or the rSK expressed in intracellularly from pET23(d)SK-NTR.

Ten ml of LB-Amp media were inoculated with *E. coli* BL21 cells harboring pET-SK-FN (4,5) and incubated at 37 °C for 12 h with shaking (200 r.p.m.). This inoculum was used to seed 200 ml of LB-Amp medium, and incubated for 2 h at 37 °C with shaking. At this time, the OD_{600} of the culture was 0.600. The production of SK-FBD(4,5) protein in this culture was then induced by the addition IPTG to 2 mM, and incubation continued for another 3 h at 37 °C with shaking. The OD_{600} had reached 1.2 by this time. The cells were harvested by centrifugation (8000 g x 20 min) at 4 °C, washed once with ST buffer, and resuspended in approx 14 ml of the same buffer over ice. This cell suspension was then subjected to sonication in the cold (20 sec pulses with 20 sec. gaps; total time 15 min). The lysate so obtained was then centrifuged (10000 g x 30 min) at 4 °C. The supernatant was carefully decanted into a separate flask. This solution contained approx. 6 mg/ml protein as estimated by Bradford's method using BSA as standard, and had a total of 1.5×10^4 I.U of SK activity as measured by the chromogenic peptide procedure (see description of methods, given above). The lysate was then split into two portions of ~6 ml each to effect either air oxidation or glutathione-mediated oxidation of the SK-FBD(4,5) polypeptide. For the air oxidation, the 6 ml lysate was diluted to a total of 40 ml of solution (reaction mixture A) which contained (final concentrations): Tris-HCl (pH 7.5), 50mM and NaCl, 150 mM. In reaction B (reoxidation using the reduced-oxidized glutathione buffer), the final volume was also 40 ml, but it also contained [besides NaCl (150mM) and Tris-Cl (50mM)], a mixture of GSSG and GSH (1:10 molar ratio, with GSH at 10 mM) and EDTA (1mM). Both reactions were subjected to gentle mixing at room temperature (24 plus/minus 2 °C) for 10 min and then passed through two separate glass columns each containing 6 ml human fibrin-Sepharose at a flow rate of 20 ml/h in a recycled mode i.e. the effluent was passaged back into the column with the use of a peristaltic pump. After 18 h of passage through the respective columns, the flow of the reaction mixtures

was terminated. Each column was then washed with 50 ml of binding buffer (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) followed by 50 ml each of 2 M urea (in binding buffer), folloed by 6 M urea in the same buffer (to effect tightly bound protein). All washings were collected in 10 ml fractions with a fraction collector, and analyzed for protein by Bradford's method, as well as SK activity using chromogenic substrate and human plasminogen as substrates was also determined for each fraction. The total yield of protein in the 6 M urea washings was 280 ug in the case of reaction A (air oxidation), and 380 ug in case of Reaction B (GSG-GSSH mediated refolding). These two pools represented, respectively, the fibrin-binding SK-FBD(4,5) protein obtained after air oxidation or glutathione-catalyzed refolding of the intracellular protein expressed from the plasmidpET23(d)SK-FBD(4,5). The specific activity of both the fibrin-Sepharose binding protein fractions from Reaction A and B were found to be almost identical (2.5×10^4 IU/mg and 2.1×10^4 I.U./mg, respectively). The dilute protein fractions were concentrated approx. 10-fold with centrifugation in Sartorius centristart filters. The concentrated fractions were then analyzed by SDS-PAGE, with and without reduction with beta-mercaptoethanol. On reducing SDS-PAGE (i.e. with beta-mercaptoethanol treatment of the samples), the reoxidized-refolded SK-(FN4,5), irrespective of the method of reoxidation, showed a single predominant band, but one with higher MW (~57 kD) as compared to the native SK standard (47 kD) as expected on the basis of the chimeric design of the hybrid gene. Essentially the same patterns were obtained when the SDS-PAGE was conducted without beta-mercaptoethanol, a treatment wherein any of the cystine S-S bridges would not be reduced to cysteine -SH groups. This indicated that the refolded molecules contained essentially monomeric intra-molecular S-S bonds, and contained negligible quantities of higher molecular weight (i.e. polymeric) products formed due to inter-molecular S-S bridge formation between the SK (FN4,5) molecules as a result of the rexodiation step. When analyzed by the Ellman DTNB color reaction for thiol groups (Habeeb, A.F.S.A., 1972., *Methods in Enzymol.* 25:457., Academic Press, New York) these fractions showed the complete absence of any free -SH group, indicating that the oxidation of the original 8 cysteines present in the reduced form of the chimeric polypeptides, to S-S bridges, was complete as a result of the reoxidation/refolding step.

Example 4. Construction of a hybrid gene between SK and FBD pair 1 and 2, and cloning and expression of the chimeric polypeptide in *E. coli*.

The construction of a hybrid gene between SK and FBD pair 1 and 2 involved a strategy closely similar to that utilized for hybrid construction between SK and FBD pair 4 and 5 (Fig. 18). The essential 'units' used in both the constructs were similar i.e., DNA encoding residues 1 to 383 of the SK gene, a polyglycine linker between the two genes, and a TG recognition site for cross-linking, removal of the stop codon of the SK gene and introduction of a new stop codon at the end of the FBD segments [either FBD(4,5) or FBD(1,2) etc]. This strategy also exploited the use of the Bsm I site of the SK gene as a common junction-point for the fusion between the SK and FBD(1,2) gene segments. However, the strategy differed from that employed for constructing SK-FBD(4,5) fusion in that the amplification of the FBD (1,2) domains was carried out in one stage, unlike that

of SK-FBD(4,5) wherein two consequetive PCRs with differing 5'-primers were utilized (Example 3). This was because in case of the SK-FBD(1,2) construct, a very large primer was not required as a TC recognition site is naturally present in the FN gene just at the begining of the FBD-1 domain (Cf. Fig. 6), thereby obviating the need to engineer a TG site in the upstream primer.

The FBD pair (1,2) was amplified from the plasmid pFHMG-60 (containing all five of the FBD encoding sequences of human fibronectin) with the following two primers whose sequences are provided below. Also shown are the 5'- ends containing non-hybridizing sequences (in bold letters) and the incorporated RE sites therein to facilitate cloning and/or docking into the SK gene; the areas hybridizing with the target DNA sequences in the templates are also underlined.

Upstream primer, MY-10

SK sequence (codons 377-383; Cf. Fig. 3)

5'- G-TAC-GGA-TCC G-AAT-GCT-AGC-TAT-CAT-TTA-GCG-GGT-GGT-GGT- CAG-GCG-CAG-CAA-ATG-GTT- 3'

Bam HI Bsm I (-gly-gly-gly-) hybrdizes at thr TG -recognitn. site just before the FBD sequences (Cf. Fig. 6)

Downstream primer, MY-6

5'- GGC- CTT-AAG- AGC-GCT-CTA-TTA- GAT-GGT-ACA-GCT-TAT-TCT-3'

'clamp' Eco RI site Eco 47 III Stop codons sequence hybridizing with FBD(1,2) codons 99-104 (Cf. Fig. 6)

The following conditions were used for the PCR: Each of the dNTP's: 200 uM; pFHMG-60 vector as template : 1 ng; MY-10 and MY-6 primers: 20 pmol each; pfu polymerase : 2.5 units; 1X pfu polymerase buffer (Stratagene Inc.), and a total reaction volume of 100 ul. A 'hot start' was given for 5 min at 94 °C. The following cycling parameters were used : denaturation at 92 °C for 45 sec, annealing at 50 °C for 60 sec, and extension at 72 °C for another 60 sec. A total of 30 cycles were given, after which a final extension was provided at 72 °C for 10 min. The amplification of the SK-FBD (1,2) hybrid cassette was checked by loading one-tenth of the reaction mixture on a 2% agarose gel. This demonstrated that the expected DNA (369 bp) was satisfactorily amplified in the absence of any background bands. The amplified PCR product was then cloned into pBluescriptII KS(-) after purification through Qiagen PCR-purification column using the manufacturer's protocol. Approximately 2 ug of pBluescriptII KS(-) vector was digested with 10 units of Sma I restriction enzyme in 1X NEB-4 buffer in a 20-ul reaction by incubating the digestion mixture at 25 °C for 12 h. The enzyme was inactivated by heating at 55 °C for 5 min and the linearized plasmid DNA was purified after electrophoresis on a 1% agarose gel. Then, 150 ng of the SmaI-digested vector was ligated with approx. 120 ng of the purified insert DNA (PCR product) in a 25-ul reaction after adding 2.5 ul of 10X (stock) ligase buffer (New England Biolabs Inc., MA, USA), 1ul of 10 mM rATP stock and 400 weiss units of T4 DNA ligase. The ligation was done by incubating the reaction tube at 16 °C for 12 h. After the ligation, the ligase was

inactivated by heating at 70 °C for 10 min. The DNA in the ligation mixture was precipitated with n-butanol and then dissolved in 20 ul of dist. water and approx. one-third used to transform *E. coli* XL-Blue electrocompetent cells (Stratgene Inc., USA) by electroporation. Transformants were selected by plating on LB-Amp plates. Miniprep plasmid DNA was prepared from eight selected clones and analysed by agarose gel electrophoresis. The plasmid DNAs of the transformants were run alongwith pure pBluescript II KS(-) to identify positive clones with larger MW, signifying the presence of the PCR-generated insert DNA. Three transformants were found to be moving slower than the pBluescript DNA on 1.2 % agarose electrophoresis. To further confirm that these contained the DNA insert, their plasmid DNAs were digested with EcoRI and BamHI enzymes since EcoRI and BamHI were two of the sites that were introduced in the PCR product during amplification. This showed that a 370-bp fragment, corresponding to the size of the PCR product was liberated, clearly establishing that these clones contained the desired cassette. This was finally confirmed by automated DNA sequencing by the Sanger di-deoxy chain-termination method which showed a complete correspondence with the sequence expected on the basis of the primers and the target DNA viz., FBD(1,2) alongwith a short stretch at its 5'-end carrying SK-specific and intergenic sequences. The sequencing also established the absence of any other mutation in the amplified DNA. The cassette subcloned in pBluescriptII KS(-) was then transferred into the SK-containing vector, pSKMG400 , in order to fuse it in-frame with the SK ORF utilising the common Bsm I site. For cloning the SK-FBD(1-2) hybrid cassette into pSKMG400 vector, both vector and insert DNAs were first digested with BamHI. Roughly 2ug of the pBluescript-FBD(1,2) and 4 ug of the pSKMG400 were digested with 8 units each of the BamHI enzyme in a 30 ul reaction utilizing buffer D of Promega. The tubes were incubated at 37 °C for 6 h. A small aliquot was run on a 0.7 % agarose gel to check for the digestion. After confirming completion of digestion, the reaction was stopped by adding 0.1 volume of 100 mM EDTA. The digested samples were loaded onto 0.8 % agarose gel and the desired fragments were cut out as agarose blocks. The DNA was extracted by treatment with beta-agarase as detailed before, and quantitated. Ligation reaction was set up between double-digested vector and the fragment containing the SK-FBD(1,2) cassette using ~200 ng of the vector and 30 ng of the fragment, 4 ul of the ligase buffer, 4 ul of 10 mM rATP, and ~600 Weiss units of ligase in a total volume of 40 ul. The ligation reaction was incubated at 16 °C for 12 h. The ligase was inactivated by heating the tube at 70 °C for 10 min then the ligated DNA was precipitated using n-butanol, air-dried and dissolved in a small volume of sterile distilled water. For the transformation step, approx. 100 ng of the ligated DNA was used to transform *E. coli* XL-1 Blue electrocompetent cells which were plated on LB-Amp plates. Five colonies were picked up and used for plasmid minipreparations. The plasmid DNAs were digested with Afl II and Eco47 III restriction enzymes separately. pBluescript FBD(1,2) and pSKMG400 vectors digested with the same enzymes were kept as controls. The digestion mixtures were run on a 0.7% agarose gel along with double-digested controls. Two clones showed linearization upon Eco47 III digestion. The pBluescript FBD(1,2) control also showed linearization with Eco 47 III digestion, as expected. However, the positive clones were of higher molecular size due to the presence of SK. The pSKMG400 did not show any digestion with Eco 47 III enzyme. The positive clones also gave out an insert upon AflII digestion,

as anticipated from the known presence of a single Afl II site in SK and another in the FBD(1,2) segment.

For the expression of the hybrid SK-FBD(1,2) polypeptide, the Bsm I-Not I fragment from pSKMG400-FBD(1,2) was transferred into pET23(d)SK-NTR at the same sites (see Fig. 19a). Approximately 10 ug of pSKMG400-FBD(1,2) plasmid DNA was digested with 15 U of Not I enzyme in NEB-3 buffer supplemented with 1X BSA by incubating at 37 °C for 6 h in a 60-ul reaction. A second addition of Not I enzyme was again made and the reaction mixture was further incubated for another 6 h. A small aliquot was removed to check for the completion digestion by running an agarose gel. After the NotI digestion, the DNA was precipitated with ethanol and sod. acetate (0.3 M), redissolved in 20 ul of dist. water and digested with 20 U of BsmI enzyme in NEB-2 buffer in a 80-ul reaction at 65 °C for 14 h after overlaying the reaction mixture with 50 ul of mineral oil to avoid evaporation. Similarly, in parallel, approx. 5 ug of the vector (pET23(d)SK-NTR) was double-digested with 20 U of Not I and 15 U of BsmI enzymes, sequentially. The linearized vector, and insert were isolated by running a 1% agarose gel and loading the above-mentioned digestion mixtures in well-separated wells.The vector and insert bands were cut out from the agarose gel using a clean scalpel and the respective DNA fragments were purified, quantitated spectrophotometrically and ligated at a molar ratio of 1:5 of vector: insert . Approximately 600 ng of the BsmI/NotI double-digested vector was ligated with around 250 ng of the BsmI/NotI double-digested insert in a 20-ul reaction by adding 600 Weiss units of ligase (NEB) and 2 ul of 10 x ligase buffer (also of New England biolabs, Inc.) and incubating for 14 h at 16 °C.. The ligation mix was then heat-inactivated at 70 °C and 15 min, and the DNA was n-butanol precipitated, air-dried, dissolved in 20 uL of sterile water and approx. one-thirds directly used to electroporate *E. coli* XL-1 Blue electrocompetent cells. The transformants were selected on LB-Amp plates. Ten transformants were picked up and inoculated into fresh LB-Amp for plasmid minipreparation. The miniprep DNAs were digested with BsmI and NotI enzyme alongwith pSKMG400-FBD(1,2) as control. Three clones were positive in terms of liberating an insert equal in size to that of the insert liberated from pSKMG400- FBD(1,2).One of the clones was then sequenced to confirm the nucleotide sequence of the SK-FBD gene (see Fig.19b). The plasmid DNA for this clone was transformed into *E. coli* BL-21 strain, grown in liquid culture and induced for the expression of SK-FBD(1,2) hybrid protein with IPTG, as descibed earlier. Cells from 1.5 ul of the induced culture were pelleted down by centrifugation and were lysed in 100 ul modified SDS-PAGE sample buffer ; approx. 25 ul lysate, alongwith that from cells harbouring pET23(d) SK-NTR as control was analysed on 10% SDS-PAGE gel. In parallel, cultures were also grown where IPTG addition was omitted (uninduced controls) and similarly analysed alongwith induced cultures by SDS-PAGE. The gels showed distinct bands of 57 kD in the IPTG-induced cultures (roughly representing 20 % of the soluble protein fraction) indicating that the hybrid SK-FBD(1,2) fusion protein had been expressed at high levels intracellularly. In the case of parallel pET23(d)SK-NTR harboring cultures, a major band corresponding to 47 kD, the position of native SK was observed.

Example 5. In-frame fusion of DNA segments encoding FBD(4,5) at the N-terminal end of the ORF encoding for SK and cloning and expression of the hybrid gene-construct FBD(4,5)-SK in *E. coli*.

The construction of FBD(4,5)-SK hybrid was accomplished by the splicing overlap extension (SOE) method, a procedure in which two (or more) DNA fragments are joined together employing PCR , without using either DNA scission or ligation (in this context, reference may be made to the following publications: Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R., 1989, *Gene* 77:61). The two fragments to be joined by SOE need to have mutually complementary sequences at their respective junctions where the joining is to take place so as to form an 'overlap' (see Fig. 20). These regions of complementarity can be engineered into the two DNA fragments, or 'blocks', to be joined (in the present case, the SK and FBD sequences) through separate PCRs each employing primers specially designed for this purpose. These two PCR-generated blocks are then used in the overlap extension reaction, in a third PCR, wherein the complementary strands hybridize partially at their 3'-ends through the regions of mutual complementarity after strand separation (denaturation) and reannealing. Thus, these two DNA strands act as megaprimers on each other, and in the presence of thermostable DNA polymerase, the 3'-ends of this intermediate are extended to form the full-length (i.e. fused) segment, which may then be further amplified using the flanking primers derived from the first two PCRs used to generate the two DNA blocks. The FBD-SK fusions were made using four synthetic oligonucleotide PCR primers viz., KRG-8, KRG-9, KRG-11 and KRG-12 whose sequences and design are described below (see also Fig. 19 for the overall scheme followed for the construction of the chimeric gene-construct).

Upstream PCR-I primer KRG-8:

Transglutamate recognition site

150 152 154

5'-CC-ATG- GTG-CAA-GCA-CAA-CAG-ATT-GTA-CCC-ATA-GCT-GAG-AAG-TGT-3'

Partial Nco I site

hybridizes to begining of FBD(4) segment (codon numbers of FBD are shown as per Fig. 6)

Downstream PCR-I primer KRG-9:

sequence complementary to codons 1-5 of SK (No.'s indicated below)

5'- CTC-AGG-TCC-AGC-AAT -ACG-AAC-ATC-GGT-GAA-GGG-GCC-AGA-T-3'

5 4 3 2 1 259 257 255 253

sequence hybridizing with end of FBD(5) segment (No.'s indicated are codons, as per Fig. 6)

Upstream PCR-II primer, KRG-11

FBD(5) sequence, as overhang; Codon No.'s (Cf. Fig. 6) are shown.

sequence hybridizing with SK gene; codon No.s (Cf. Fig. 3) are indicated.

5'-TTC-ACC-GAT-GTT-CGT- ATT-GCT-GGA-CCT-GAG-TGG-CTG-CTA-GAC-3'
255 257 259 1 3 5 7 9

Upstream PCR-II primer, KRG-12

5'-TGG-TTT-TGA-TTT-TGG-ACT-TAA-GCC-TTG-3'
62 60 58 56 54

Note: sequence hybridizing with SK gene (codon No.'s are indicated; see Fig. 3)

As can be seen above, the upstream primer, KRG-8, was homologous to the the begining 18 nucleotides of the 'anticoding' strand of the FBD-4 encoding DNA and also carried at its 5' end non-hybridizing DNA sequences that encoded for a Nco I site (to facilitate the 'docking' of the SOE product into the Nco I site of the expression vector, thus recreating the ORF for the FBD(4,5)-SK fusion sequences). The upstream primer also contained sequence coding for a transglutaminase site. The downstream primer KRG-9 was designed to hybridize with the end of the FBD(5) DNA sequence, but also contained at its 5' non-hybridising end, nucleotides complementary to the first 5 codons of the ORF encoding mature *S. equisimilis* SK (see Fig. 6). The template used for the first PCR to obtain Block I (see Fig. 20) was FBD(4,5) cloned in pBlueScript [pSKMG400-FBD(4,5)]. The first PCR (termed PCR-I) was carried out using approx. 20 ng template and 30 pmol of each primer in a 100 uL-reaction using the buffer provided by Stratagene Inc., the supplier of the *pfu* thermostable polymerase. The PCR employed 25 cycles with the following conditions: 94 °C for 45 sec (denaturation step) followed by 50 °C for 1 min (annealing step), and 72 C for 1 min (extension step). This was followed by an incubation for 4 min at 72 °C for extension of any incomplete chains. The PCR resulted in the generation of a single species of DNA, in accordance with the size expected from the fusion construct (368 bp), as observed by agarose gel electrophoresis; this DNA species was isolated from the gel as a small agarose block, and subjected to further purification using the agarase treatment method, described earlier.

For obtaining the DNA Block II for the SOE reaction, only the region of the SK gene corresponding to nucleotide 1 to 186 (approx. corresponding to the first 63 amino acid residues of SK; see Fig. 3) was amplified using pSKMG400 as template in PCR II, using the primer set KRG 11 (upstream primer) and KRG 12 (downstream primer). This region encompasses the unique Afl II site in the SK gene (see Fig. 4). The upstream primer contained non-hybridizing bases that were homologous to the last five codons of the FBD(5) -encoding DNA (viz., codons 255-259), followed by a stretch of bases hybridizing to the first 27 bases of the anti-sense strand of the SK

ORF (see Fig. 3). The downstream primer contained sequences hybridizing with the stretch of DNA encoding for residues 55-63 of SK containing the Afl II restriction site so that the SOE product could be docked back into the full-length SK gene contained in the vector used for the expression of the hybrid gene(see Fig. 21a). The PCR was carried out essentially as described for PCR I, above, except that 90 ng of template was chosen and the cycling conditions selected had a lower annealing temp. (43 °C) dictated by a relatively lowered T_m of one of the primers. The PCR gave a single DNA band of the expected size (201 bp) on agarose gel electrophoresis, which was isolated and purified as for PCR I product (Block I). Splice overlap extension reaction (PCR III) was then carried out to obtain the hybrid DNA between the FBD and SK ORFs. In this reaction, approx. equivalent amounts of the purified DNAs from PCR I and PCR II were mixed together (representing approx. one-fifteenth of the amplified DNA obtained from PCRs I and II) in a 100-uL reaction. To bring about optimal and specific annealing between the hybridizing areas of the two partially complementary strands from the FBD(4,5) and SK 1-63 DNA blocks (see Fig. 20) (Phase 1), the reaction was first carried out in the absence of any other primers, using *pfu* DNA polymerase and the buffer specified by its supplier, employing the following conditions: 98 °C for 2 min, slow temperature decrease (i.e. 'ramp' of 4 min) to 50 °C, maintainance at 50 °C for 1 min, followed by 3 min at 65 °C. A 'hot start' was used for the initiation of the PCR (i.e. the DNA polymerase was added into the reaction after all other components had been added and thermally equilibrated to the highest temperature in the cycle). A total of 10 cycles were carried out first (Phase I), to allow formation of overlapped extended products. In the second phase, primers KRG 8 and KRG 12 were added under hot start conditions, and another 25 cycles were given at the following cycling conditions: 94 °C for 1 min (denaturation step), 40 °C for 1 min (annealing), followed by extension at 72 °C for 1 min to amplify the fusion products. Finally, after 10 min at 72 °C, an aliquot from the PCR was analysed by agarose gel electrophoresis. It showed the clean appearance of the expected hybrid product (539 bp) with the absence of any other background bands. This was isolated from the agarose gel, purified and then kinased with T4 phage polynucleotide kinase by standard protocols. The kinased (i.e. 5'-phosphorylated) product was then blunt-end cloned at the Eco RV site of pBlueScript. Clones containing the SOE product were selected by restriction enzyme digestion to isolate the inserts and measuring their size by agarose gel electrophoresis. Two positive clones were then sequenced to confirm the identity of their DNA inserts as well as the absence of any mutations(see Fig21b). After Nco I and Afl II digestion of one of these two clones, the Nco I-Afl II fragment carrying the FBD-(4,5)-SK 'hybrid cassette' was ligated with Nco I-Afl II digested SK-expression plasmid (pET(23d)-SK) and transformation of *E. coli* XL-Blue cells was carried out to obtain the hybrid FBD(4,5)-SK ORF in this vector (Fig. 21a). This plasmid construct was then transformed into *E. coli* BL-21 cells to monitor expression of the hybrid FBD-SK construct from the T7 promoter-based vector, as described before. The SDS-PAGE gels showed the expression intracellularly of a protein with the expected MW (approx. 57 kD) at a level of around 20 percent of total intracellular, soluble proteins.

Example 6. In-frame fusion of DNA segments encoding for FBD segments 4 and 5 at both the ends of the DNA ORF encoding for SK, and cloning and expression of the hybrid gene-construct so formed, FBD(4,5)-SK-FBD(4,5), in *E. coli*.

The steps involved in the construction of a SK-FBD hybrid wherein the FBD(4,5) domains were fused in-frame simultaneously at both ends (i.e. the N- and C- termini) of the SK gene [i.e. FBD(4,5)-SK-FBD(4,5)] are shown schematically in Fig. 22a. It is based on the cloning of the FBD(4,5)-SK(1-57 residues) cassette obtained from pBlueScript-FBD(4,5)-SK vector, described in Example 5 (above) into pET-(23d)SK-FBD(4,5) at the begining of the ORF for SK. Approximately 5 ug each of pET(23 d)SK-FBD(4,5) and pBlueScriptFBD(4,5)-SK plasmid DNA were digested with Afl II and Nco I restriction endonucleases and the digests were electrophoresed on 1.2 % agarose gels alongwith standard DNA size markers by standard methods. In each case, two fragments were observed, corresponding to the vector DNAs (expected size, 5012 bp) devoid of the Nco I-Afl II fragment, and the latter fragment [approx. 520 bp in the case of pBlueScriptFBD(4,5)-SK and 140 bp in case of pET-(23d)SK-FBD(4,5)] released from the parent vectors as a result of the double-digestion (see Fig. 22a showing the Afl II and Nco I sites in the two vectors). The NcoI-Afl II fragment from pBlueScript FBD(4,5)-SK, containing the FBD(4,5)-SK(1-57 residues) cassette, to be used as insert, and the NcoI-AflII digested vector DNA from pET-(23d)SK-FBD(4,5) were isolated from the agarose gel and purified. Both fragments were then subjected to ligation using T4 DNA ligase under standard conditions using a molar ratio of 1:2 of vector to insert DNA. The ligation mixture was then transformed into electro-competent *E. coli* XL-1 Blue cells. Positive clones, with both ends of SK fused with the FBD(4,5) domains, were selected on the basis of difference in size from the parent vectors, as well as their ability to yield the expected fragment (containing FBD sequences at both ends of the insert) after digestion with Nco I and Bam HI enzymes (see Fig. 22a). The veracity of the constructs was then established by subjecting one of the selected clones to automated DNA sequencing using Sanger's di-deoxy method to sequence the entire hybrid ORF (see Fig22b). This demonstrated that the construct had the expected design and sequence, with one 'set' of FBD4,5 domain fused at the begining of the SK gene, and another at its end (i.e. after DNA encoding for residue 383). The plasmid DNA was isolated from this clone [designated pET23(d)FBD(4,5)-SK-FBD(4,5)], and used to transform *E. coli* strain BL-21 electro-competent cells, in order to express the FBD-SK-FBD hybrid constuct intracellularly in *E. coli*. The hybrid gene was then expressed in E. coli intracellularly after induction with IPTG exactly as described earlier, and the cell lysates analysed by SDS-PAGE. These showed the expression of a polypeptide of approx. MW 65 kD as expected from the incorporation of the two FBD segments at each ends of the SK (1-383) gene. The level of expression of this protein was observed to be approx. 20-25 percent of the total soluble protein fraction.

Example 7. Purification of various chimeric constructs formed between SK and FBDs after expression in *E. coli* and refolding, and testing of their affinity for human fibrin.

Fifty ml LB-Amp (containing 100 ug/ml of ampicillin per ml) were seeded with *E. coli* cells harbouring either pET23(d)-SKFBD(4,5), pET23(d)-SKFBD(1,2), pET-23(d)FBD(4,5)-SK or pET23(d)-FBD(4,5)-SK-FBD(4,5) plasmid constructs in separate flasks (150 ml capacity). The inoculation was done from the respective glycerol stocks, and the culture was incubated at 37 °C for 12 h on a rotary shaker (200 r.p.m.). This pre-inoculum was used to seed fresh LB-amp at 5 % (v/v) level (one litre total for each type of *E. coli* BL-21 cells harbouring one of the different plasmid constructs described above with 500 ml medium per 2 liter conical flask), and the cultures shaken as above at 37 °C for approx. 2 h 30 min, at which time the OD600 of the cultures had reached a value of 1.0-1.1. The expression of the chimeric SK/FBD polypeptides in the cultures were then induced by the addition of IPTG to 1 mM, and continuing further the incubation for another 3 h. Cells from all four cultures were then harvested by high-speed centrifugation (8000 g x 30 min) at 4 °C, and washed once with 500 ml cold ST buffer (pH 7.5). Finally, each cell pellet was suspended in cold 25 ml ST buffer, pH 8.0. The wet-weight of the pellets obtained from 1 liter cultures varied between 4.3-4.5 g. Each cell-suspension was then subjected to ultra-sonication to effect cell-lysis using standard methods. The lysates so obtained (approx. 28 ml each) were subjected to high-speed centrifugation at 4 °C to pellet any unlysed cells and/or cell debris. The protein conc. in the supernatants varied between 15.0 to 16.0 mg/ml. These were then diluted to a final protein of 1 mg/ml using distilled water, together with the addition of the following additional components (final concentrations in the diluted mix are given): Tris-Cl, pH 8.0, 50 mM; NaCl 150 mM; EDTA 1 mM; mixture of reduced and oxidized glutathione 123:50 mg. To these refolding mixtures (approx. 400 ml each) were then added 30 ml (packed volume) of fibrin-Sepharose beads pre-equilibrated with 50 mM Tris-Cl, pH 8.0. The mixtures were stirred at 22 °C for 16 h to effect reoxidation/refolding. The solutions were then passed through 50 ml-volume axial glass columns fitted with fritted glass disks (to retain the Sepharose-beads). The packed fibrin-Sepharose beds were then washed with approx. 170 ml binding buffer (50 mM tris-Cl, pH 8.0, and 150 mM NaCl), followed by 100 ml of 2 M urea (in binding buffer), and finally the fibrin-bound protein was eluted with 6 M urea (in binding buffer). All the washing/elution steps were carried out at a flow rate of ~30 ml/h using a peristaltic pump assembly. The chromatographic profile in case of SK-FBD(4,5) fibrin-Sepharose affinity purification, and analysis of the different fractions, are shown on Fig. 23. Similar results were obtained in case of the other SK-FBD constructs. A total of 3.8 mg of protein was eluted alongwith the 6 M urea-wash in the case of SK-FBD(4,5), whereas for SK-FBD(1,2) approx. 4 mg, for FBD(4,5)-SK 3.5 mg, and for FBD(4,5)-SK-FBD(4,5) 6.2 mg of protein was obtained at the 6 M urea elution step. In case of the SK control, no protein was found to elute alongwith the 6 M urea. The removal of the urea, and concentration of the protein, was effected by ultrafiltration through 10 kD cut-off membranes. Aliquots of each of the four fibrin-specific SK-FBD chimeric products were then subjected to SDS-PAGE analysis on 10 percent acrylamide gels with and without reduction with beta-mercaptoethanol to determine their relative purities as well as

monomeric/polymeric character. Standard molecular weight marker proteins as well as pure native SK from *S. equisimilis* were also run on the same gels. The SDS-PAGE, analysis, either in the presence or absence of reducing agent, showed all of the fibrin-Sepharose eluted chimeric product to be essentially pure and monomeric; in all cases, a single prominent band stained with Coomassie Blue and very few faint background bands were visible (not more than 2-5 % cumulatively). The MWs of the refolded proteins were completely in accord with those expected from theoretical considerations i.e. extrapolated from the MW of individual domains of FBD, the SK portion, and linker sequence, if present. The SK-FBD(4,5) and SK-FBD(1,2) bands moved with the same mobility on SDS-PAGE, with an apparent MW of around 55 kD; however, the FBD-(4,5)-SK construct showed a slightly lowered mobility as compared to either SK-FBD(4,5) or SK-FBD(1,2). This was in accord with the fact that whereas the former two hybrid constructs contained approx. 31 amino acid residues' deletion at the C-terminal end of the SK moeity of the hybrid, the FBD(4,5)-SK construct had full-length SK integrated in its design (see Examples, above). The FBD(4,5)-SK-FBD(4,5) construct, containing four FBDs alongwith SK, moved with a MW corresponding to 60 kD on SDS-PAGE. In the absence of beta-mercaptoethanol, the MW's calculated for all four hybrids were approximately the same as observed in the presence of beta-mercaptoethanol, indicating that the constructs obtained after refolding and binding with fibrin-Sepharose contained essentially monomeric forms of the polypeptides.

The specific activities of the purified proteins for PG activation, as determined by the chromogenic assay were: 2.2×10^4 I.U./mg for SK-FBD(4,5), 1.8×10^4 I.U./mg for SK-FBD(1,2), 4×10^4 I.U./mg in case of FBD(4,5)-SK and 5×10^2 I.U./mg for FBD(4,5)-SK-FBD(4,5), respectively. Under the same conditions, native *S. equisimilis* SK, or SK purified from *E. coli* (Met-SK) as described in Example 2, above, showed a much higher activity (~1.0×10^5 I.U./mg). The reason for the apparently lowered specific activities in case of the chimeric proteins was revealed when these were assayed by a single-phase, continuous spectrophotometric assay by directly determining their rates (slopes) for HPG activation by standard methods (Wohl, R.C., Summaria, L., and Robbins, K.C., 1980, *J. Biol. Chem.* 255:2005). These assays revealed that whereas native SK or *E. coli*-expressed Met-SK did not display an appreciable lag in the progress curves obtained for the PG activation reactions (less than 1 min), all of the hybrid proteins displayed significant initial periods in their PG activation profiles (varying from 7 to 25 min depending on the construct) wherein little or no plasmin formation occured. However, after the initial lags, the PG activation proceeded with a high rate, generating slopes closely similar to those obtained with native SK (see below).

Example 8. Functional characterization of the chimeric proteins in terms of their altered kinetics of plasminogen activation and fibrin clot dissolution.

The proteins prepared in Example 7, above, as well as native and Met-SK (as controls) were examined with respect to their PG activation kinetics. This essentially entailed the study of the time-course of PG activation by the various SK/FBD chimeras and the determination of their

steady-state kinetic constants for PG activation. A one-stage assay method was used to measure the activation of HPG ; reference in this context may be made to several publications in the literature e.g., Shi, G.Y., Chang, B.I., Chen, S.M., Wu, D.H. and Wu, H.L., 1994, *Biochem. J.* 304:235; Wu, H.L., Shi, G.Y., and Bender, M.L., 1987, *Proc. Natl. Acad. Sci.* 84: 8292; Wohl, R.C., Summaria, L., and Robbins, K.C., 1980, *J. Biol. Chem.* 255:2005; Nihalani, D., Raghava, G.P.S., Sahni, G., 1997, *Prot. Sci.* 6:1284). Briefly, it involved the addition of the activator proteins to be studied in a small aliquot (~5 ul) into 100 ul-volume microcuvette containing 1 uM of HPG in assay buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM chromogenic peptide substrate and 0.1 M NaCl). The protein aliquots were added after addition of all other components into the cuvette and bringing the spectrophotometric absorbance to zero. The change in absorbance at 405 nm was then measured as a function of time in a Shimadzu UV-160 model spectrophotometer (Fig.). While SK showed a rapid PG activation kinetics, the kinetics for SK-FBD chimeric protein [shown for SK-FBD(4,5) in Fig.] displayed a characteristic lag, or delay, in the initial phase of the rate of PG activation that was clearly different from the rates seen with SK. This property viz., initial delay in HPG activation, as well as its magnitude, was largely independent of the amount of the chimeric protein employed in the assay, as well as the concentration of HPG in the reaction. Another notable feature was that the lag-times associated with the different chimeric proteins under the same conditions. In the case of SK-FBD(1,2) and SK-FBD(4,5) the lag period corresponded to 10-12 min, for FBD(4,5)-SK 7-8 min, and 20-25 min in case of FBD(4,5)-SK-FBD(4,5). Under the same conditions (~1 uM HPG, 1-2 nM of protein), native SK or Met-SK displayed very little lag period (i.e. less than 1 min duration) during PG activation.

To determine the steady-state kinetic parameters for HPG activation of the activated forms of the hybrids, fixed amounts of SK or SK-FBD chimeric protein (1 nM) were added to the assay buffer containing various concentrations of HPG (ranging from 0.035 to 2.0 uM) in the 100 uL assay micro-cuvette as desribed above. The change in absorbance (representing velocity, v) was then measured spectrophotometrically at 405 nM for a period of 30 min at 22 °C. All determinations were done in triplicates and their averages taken for analysis. The kinetic parameters for HPG activation were then calculated (using the linear portion of the progress curves) from inverse, Lineweaver-Burke plots using standard procedures (Wohl, R.C., Summaria, L., and Robbins, K.C., 1980., *J. Biol. Chem.* 255: 2005), wherein the $1/v$ value is plotted on the ordinate axis and 1/S value is plotted on the abscissa, S representing the (varying) concentration of substrate (HPG) employed for the reaction/s. From these plots, the K_m for HPG (K_{plg}) and maximal velocities (at saturating HPG concentrations) were determined (set forth in the following Table).

These data clearly show that once fully activated after completion of the initial lag, all the chimeric constructs became significantly active in terms of their PG activation abilities in comparison to SK.

Table

Steady-state kinetic parameters for HPG activation by SK and SK-FBD hybrid proteins*

Activator	K_{plg} (uM)	Maximal activity#
nSK	0.14 ± 0.02	100.0
Met-SK	0.18 ± 0.01	95 ± 5
SK-FBD(4,5)	0.15 ± 0.02	52 ± 4
SK-FBD(1,2)	0.18 ± 0.03	58± 5
FBD(4,5)-SK	0.16 ± 0.02	65 ± 4
FBD(4,5)-SK-FBD(4,5)	0.20 ± 0.03	45 ± 4

*The parameters were calculated from the linear phases of the reaction progress curves after the abolishment of the lag phases. #Expressed relative to native SK from *S. equisimilis*, taken as 100 percent.

In a separate series of experiments, the rates of proteolytic dissolution of radiolabelled fibrin clots *in vitro* was examined to test whether, like native SK, the SK-FBD chimeric proteins could also efficiently break down fibrin to soluble products, a fundamental biological property of all thrombolytic agents, and also to examine if the altered PG activation kinetics observed with synthetic peptide substrate, described above (i.e. slow initial rates, followed by rates close to those observed for native SK) were also reflected at the level of clot lysis.

Radioactive fibrin clots were first prepared by mixing 400 ul of cold fibrinogen (2.5 mg/ml stock) with 50 ul of ^{125}I-labelled fibrinogen containing 9 X10^5 cpm (specific activity 7.2 x 10^5 cpm/ug protein) and adding to a solution (150 ul) containing 100 ug HPG and 0.25 N.I.H. units of thrombin (Sigma). All solutions were made in 0.1 M citrate phosphate buffer, pH 7.5, containing 0.8 percent BSA (BSA-citrate buffer). The final volume of the clotting reaction was adjusted to a total volume of 1 ml with BSA-citrate buffer. The clot was formed by incubating the mixture in a glass tube at 37 °C for 2 min. The clot was then washed thrice with 2 ml of TNT

buffer (50 mM Tris-Cl buffer, pH 7.5, containing 38 mM NaCl and 0.01 percent Tween-80) for 3 min at 37 °C. When required, non-radioactive fibrin clots were prepared exactly as described above but omitting the inclusion of ^{125}I-labelled fibrinogen from the clotting mixture. The effect of the thrombolytic agent (native SK or SK-FBD hybrid) was then studied in terms of release of radioactivity from the clot kept either in a plasma milieau or in presence of excess human fibrinogen as described below.

Clot lysis of pre-formed fibrin clots suspended in human plasma was carried out by suspending ^{125}I-labelled and extensively washed clots in 2 ml citrated human plasma, prewarmed at 37 °C, and adding different amounts of either SK or a given SK-FBD hybrid protein. The reaction tubes were rotated slowly at 37 °C in a water bath and 0.1 ml aliquots of the soluble fraction were removed at regular intervals to measure the ^{125}I-fibrin degradation products released by measuring the amount of radioactivity using a gamma counter. The total radioactivity in each clot was determined by measuring the radioactivity of the respective tube before withdrawing any aliquot prior to the addition of thrombolytic agent. A comparison of the dissolution kinetics of radio-labelled fibrin clots by native SK and the various SK-FBD chimeric proteins in plasma milieau also clearly showed that the lag displayed by the latter during the PC activation assays was essentially preserved during clot lysis also. While SK caused relatively rapid dissolution of the fibrin and a plateauing of the dissolution reaction at or around 15 min, a prolonged lag in the case of the SK-FBD(4,5) hybrid (protein approx. 10 min) was evident at the same protein concentration (representative data for these two proteins are shown in Fig. 24). In the case of the other hybrids, the lag-times in plasma were essentially as seen with PC activation assays viz., 10 min for SK-FBD(1,2), 8 min for FBD(4,5)-SK, and 18 min for FBD(4,5)-SK-FBD(4,5).

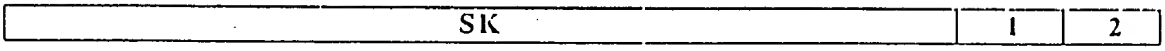
Clot lysis in the presence of an excess of human fibrinogen was also carried out by measuring the rate of dissolution of radio-labelled fibrin clot by SK or SK-FBD protein in the presence of various concentrations of human fibrinogen (1-4 mg/ml) and 100 nM of either SK or SK-FBD hybrid protein. Clot lysis was also performed in the presence of fixed fibrinogen concentration (2 mg/ml) but employing different concentrations of SK /SK-FBD protein (ranging from 50 to 200 nM). The reactions were incubated at 37 °C in a water bath with gentle shaking, and the release of ^{125}I-fibrin degradation products as a function of time was measured in the supernatant, as described above. All of the hybrid proteins were able, like SK, to dissolve the fibrin clots in a dose-dependent manner; however, there was a distinct lag in the case of the SK-FBD hybrids closely similar to that seen with clot lysis in plasma milieau. The lag period varied with construct design viz., in case of SK an absence of any appreciable lag was observed (less than 2 min). The lag times for SK-FBD(4,5) and SK-FBD(1,2) were 10-11 min; for FBD(4,5)-SK 7-8 min; and 18-20 min for FBD(4,5)-SK-FBD(4,5).

Dated 24th the day of Dec. 1998

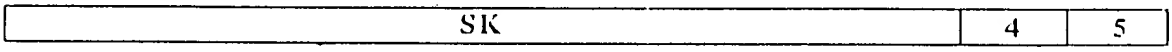
A. K. BOSE
Scientist
IPM Division, CSIR
14 Satsang Vihar Marg.

Fig. 1

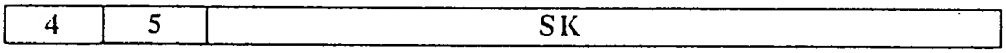
(A) FBD(1,2) fused at the C-terminal of SK



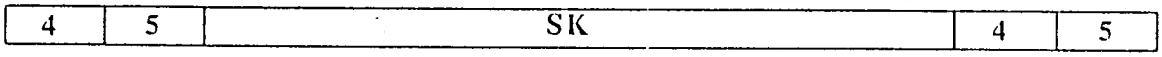
(B) FBD(4,5) fused at the C-terminal of SK



(C) FBD(4,5) fused at the N-terminal of SK



(D) FBD(4,5) fused at both the C as well as N-terminals of SK

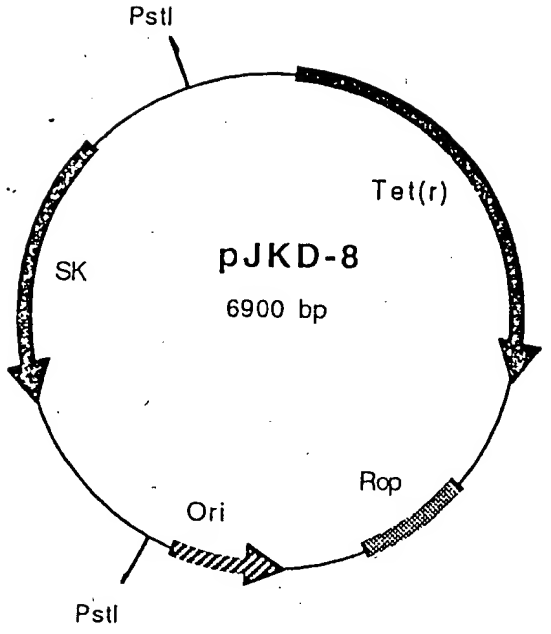


ORIGINAL

3825 DEL 98

24 DEC 1998

Fig. 2



ORIGINAL

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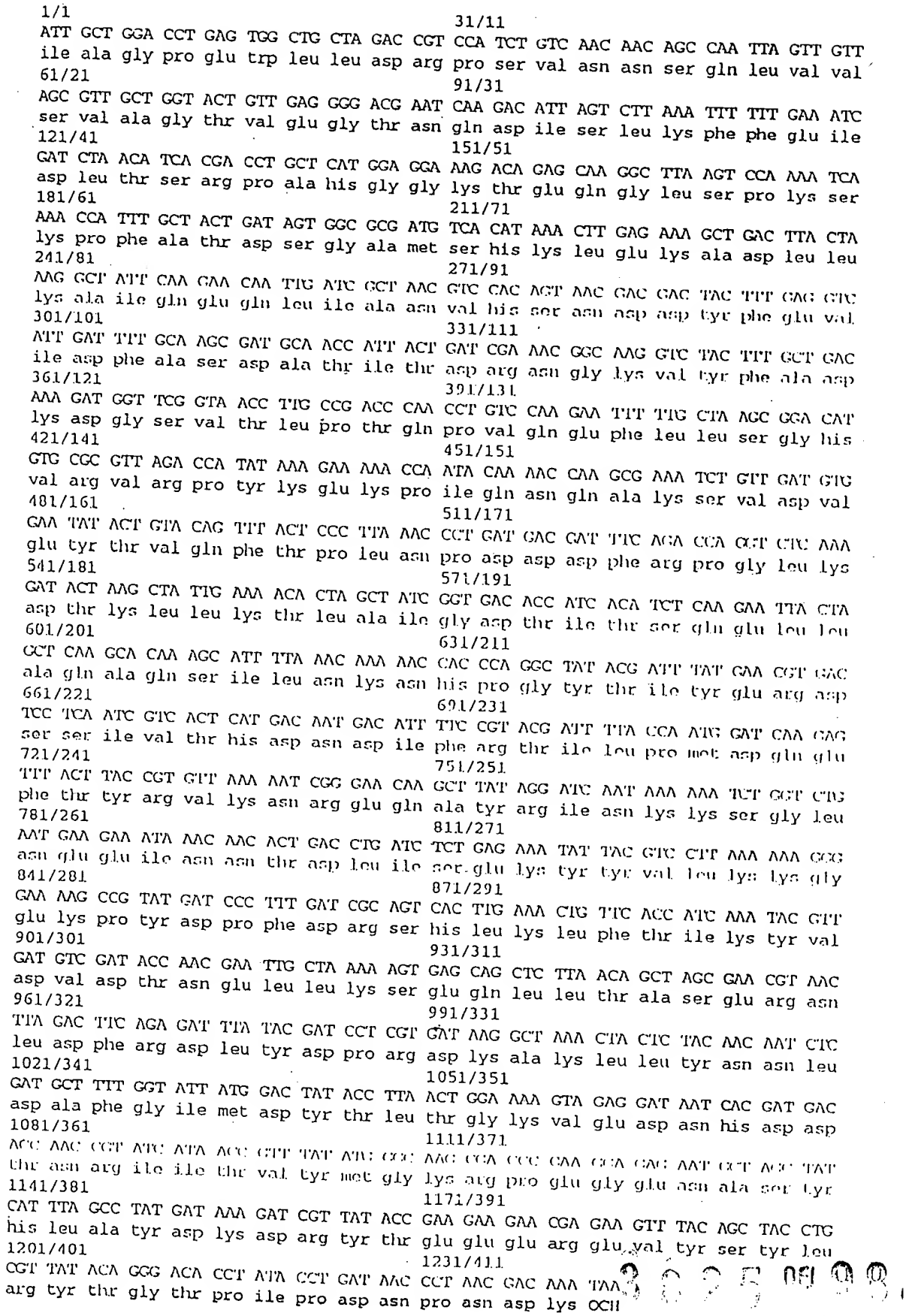

ORIGINAL

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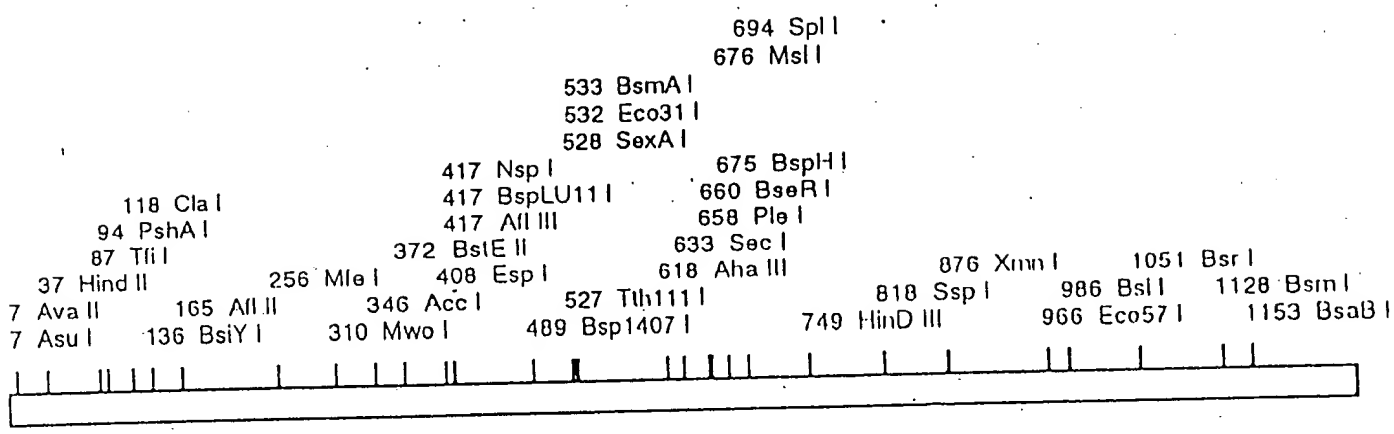
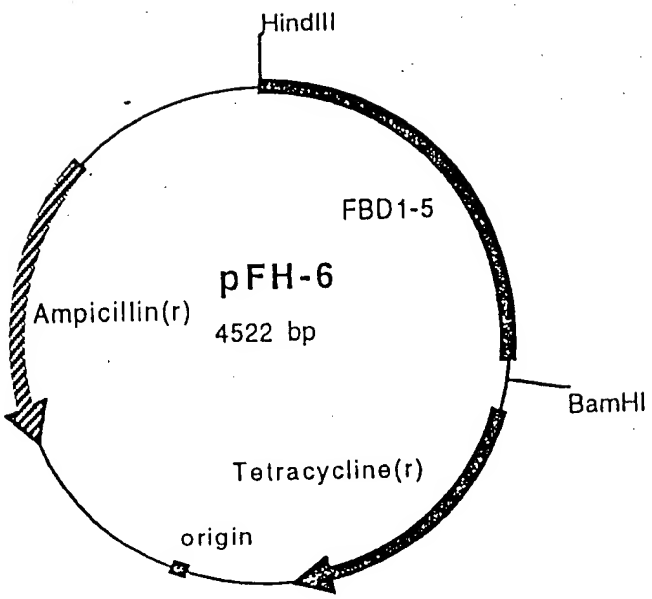


ORIGINAL

Fig. 5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pFH-6
4522 bp
Ampicillin(r)
BamHI
Tetracycline(r)
origin

Fig. 6

```
1/1                                                                 31/11
CAG GCT CAG CAA ATG GTT CAG CCC CAG TCC CCG GTG GCT GTC AGT CAA AGC AAG CCC GGT
gln ala gln gln met val gln pro gln ser pro val ala val ser gln ser lys pro gly
61/21                                                               91/31
TGT TAT GAC AAT GGA AAA CAC TAT CAG ATA AAT CAA CAG TGG GAG CGG ACC TAC CTA GGT
cys tyr asp asn gly lys his tyr gln ile asn gln gln trp glu arg thr tyr leu gly
121/41                                                              151/51
AAT GTG TTG GTT TGT ACT TGT TAT GGA GGA AGC CGA GGT TTT AAC TGC GAA AGT AAA CCT
asn val leu val cys thr cys tyr gly gly ser arg gly phe asn cys glu ser lys pro
181/61                                                              211/71
GAA GCT GAA GAG ACT TGC TTT GAC AAG TAC ACT GGG AAC ACT TAC CGA GTG GGT GAC ACT
glu ala glu glu thr cys phe asp lys tyr thr gly asn thr tyr arg val gly asp thr
241/81                                                              271/91
TAT GAG CGT CCT AAA GAC TCC ATG ATC TGG GAC TGT ACC TGC ATC GGG GCT GGG CGA GGG
tyr glu arg pro lys asp ser met ile trp asp cys thr cys ile gly ala gly arg gly
301/101                                                             331/111
AGA ATA AGC TGT ACC ATC GCA AAC CGC TGC CAT GAA GGG CGT CAG TCC TAC AAG ATT GGT
arg ile ser cys thr ile ala asn arg cys his glu gly gly gln ser tyr lys ile gly
361/121                                                             391/131
GAC ACC TGG AGG AGA CCA CAT GAG ACT GGT GGT TAC ATG TTA GAG TGT GTG TGT CTT GGT
asp thr trp arg arg pro his glu thr gly gly tyr met leu glu cys val cys leu gly
421/141                                                             451/151
AAT GGA AAA GGA GAA TGG ACC TGC AAG CCC ATA GCT GAG AAG TGT TTT GAT CAT GCT GCT
asn gly lys gly glu trp thr cys lys pro ile ala glu lys cys phe asp his ala ala
481/161                                                             511/171
GGG ACT TCC TAT GTG GTC GGA GAA ACG TGG GAG AAG CCC TAC CAA GGC TGG ATG ATG GTA
gly thr ser tyr val val gly glu thr trp glu lys pro tyr gln gly trp met met val
541/181                                                             571/191
GAT TGT ACT TGC CTG GGA GAA GGC AGC GGA CGC ATC ACT TGC ACT TCT AGA AAT AGA TGC
asp cys thr cys leu gly glu gly ser gly arg ile thr cys thr ser arg asn arg cys
601/201                                                             631/211
AAC GAT CAG GAC ACA AGG ACA TCC TAT AGA ATT GGA GAC ACC TGG AGC AAG AAG GAT AAT
asn asp gln asp thr arg thr ser tyr arg ile gly asp thr trp ser lys lys asp asn
661/221                                                             691/231
CGA GGA AAC CTG CTC CAG TGC ATC TGC ACA GGC AAC GGC CGA GGA GAG TGG AAG TGT GAG
arg gly asn leu leu gln cys ile cys thr gly asn gly arg gly glu trp lys cys glu
721/241                                                             751/251
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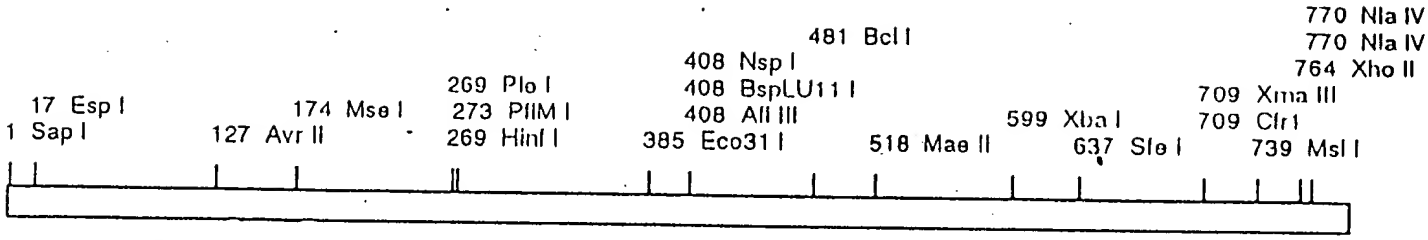
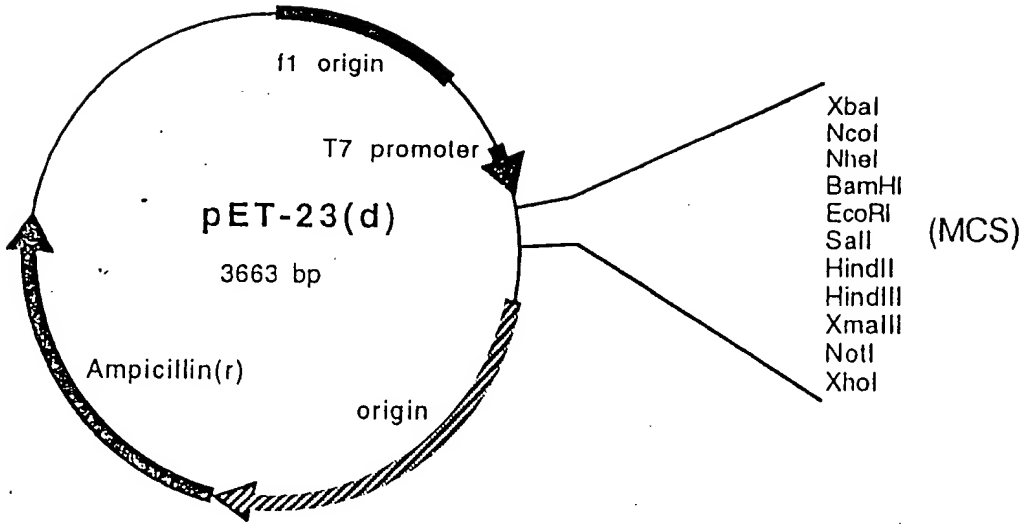
Fig. 7



FBD 1-5.DNA 790 base pairs Unique Sites

Fig. 8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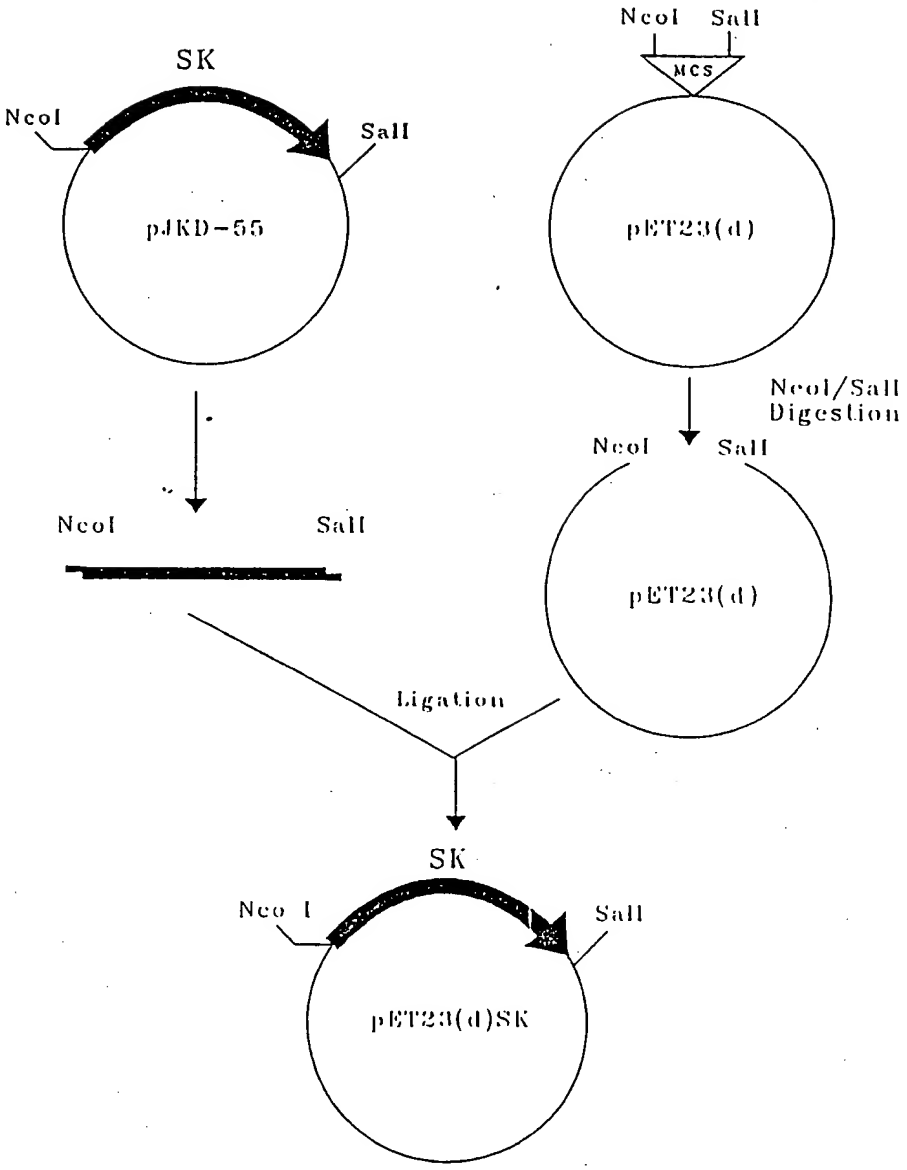
T7 promoter
pET-23(d)
3663 bp
Ampicillin(r)
origin
XbaI
NcoI
NheI
BamHI
EcoRI
SalI
HindII
HindIII
XmaIII
NotI
XhoI
(MCS)

Fig. 9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NcoI
SalI
pJKD-55
NcoI
SalI
MCS
pET23(d)
NcoI/SalI
Digestion
NcoI
SalI
pET23(d)
NcoI
SalI
Ligation
SK
Nco I
SalI
pET23(d)SK

Fig. 10

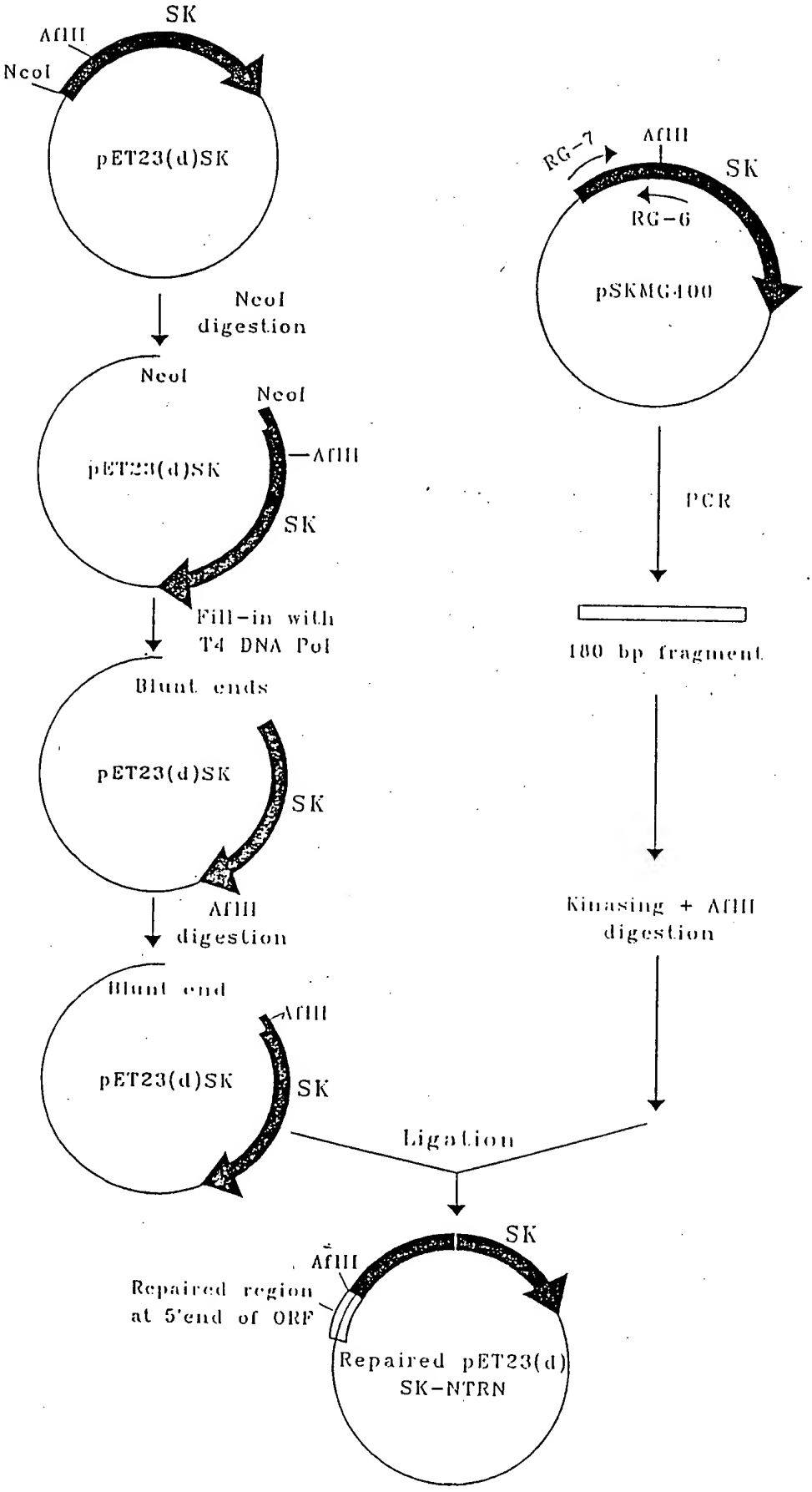
SK
AflII
NcoI
pET23(d)SK
NcoI
digestion
NcoI
NcoI
pET23(d)SK
AflII
SK
Fill-in with
T4 DNA Pol
Blunt ends
pET23(d)SK
SK
AflII
digestion
Blunt end
AflII
pET23(d)SK
SK
RG-7
AflII
SK
RG-6
pSKMG400
PCR
180 bp fragment
Kinasing + AflII
digestion
Ligation
SK
AflII
Repaired region
at 5'end of ORF
Repaired pET23(d)
SK-NTRN

Fig. 11

	10	20	30	40	50
	GCACCCGTGG	CCAGGACCCA	ACGCTGCCCG	AGATCTCGAT	CCCGCGAAAT
51	TAATACGACT	CACTATAGGG	AGACCACAAC	GGTTTCCCTC	TAGAAATAAT
101	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGATTGCTGG	ACCTGAGTGG
151	CTGCTAGACC	GTCCATCTGT	CAACAACAGC	CAATTGGTTG	TTAGCGTTGC
201	TGGTACTGTT	GAGGGGACGA	ATCAAGACAT	TAGTCTTAAA	TTTTTTGAAA
251	TCGATCTAAC	ATCACGACCT	GCTCATGGAG	GAAAGACAGA	GCAAGGCTTA
301	AGTCCAAAAT	CAAAACCATT	TGCTACTGAT	AGTGGCGCGA	TGTCACATAA
351	ACTTGAGAAA	GCTGACTTAC	TAAAGGCTAT	TCAAGAACAA	TTGATCGCTA
401	ACGTCCACAG	TAACGACGAC	TACTTTGAGG	TCATTGATTT	TGCAAGCGAT
451	GCAACCATTA	CTGATCGAAA	CGGCAAGGTC	TACTTTGCTG	ACAAAGATGG
501	TTCGGTAACC	TTGCCGACCC	AACCTGTCCA	AGAATTTTTG	CTAAGCGGAC
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601	TCTGTTGATG	TGGAATATAC	TGTACAGTTT	ACTCCCTTAA	ACCCTGATGA
651	CGATTTCAGA	CCAGGTCTCA	AAGATACTAA	GCTATTGAAA	ACACTAGCTA
701	TCGGTGACAC	CATCACATCT	CAAGAATTAC	TAGCTCAAGC	ACAAAGCATT
751	TTAAACAAAA	ACCACCCAGG	CTATACGATT	TATGAACGTG	ACTCCTCAAT
801	CGTCACTCAT	GACAATGACA	TTTTCCGTAC	GATTTTACCA	ATGGATCAAG
851	AGTTTACTTA	CCGTGTTAAA	AATCGGGAAC	AAGCTTATAG	GATCAATAAA
901	AAATCTGGTC	TGAATGAAGA	AATAAACAAC	ACTGACCTGA	TCTCTGAGAA
951	ATATTACGTC	CTTAAAAAAG	GGGAAAAGCC	GTATGATCCC	TTTGATCGCA
1001	GTCACTTGAA	ACTGTTCACC	ATCAAATACG	TTGATGTCGA	TACCAACGAA
1051	TTGCTAAAAA	GTGAGCAGCT	CTTAACAGCT	AGCGAACGTA	ACTTAGACTT
1101	CAGAGATTTA	TACGATCCTC	GTGATAAGGC	TAAACTACTC	TACAACAATC
1151	TCGATGCTTT	TGGTATTATG	GACTATACCT	TAACTGGAAA	AGTAGAGGAT
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1251	CGAAGGAGAG	AATGCTAGCT	ATCATTTAGC	CTATGATAAA	GATCGTTATA
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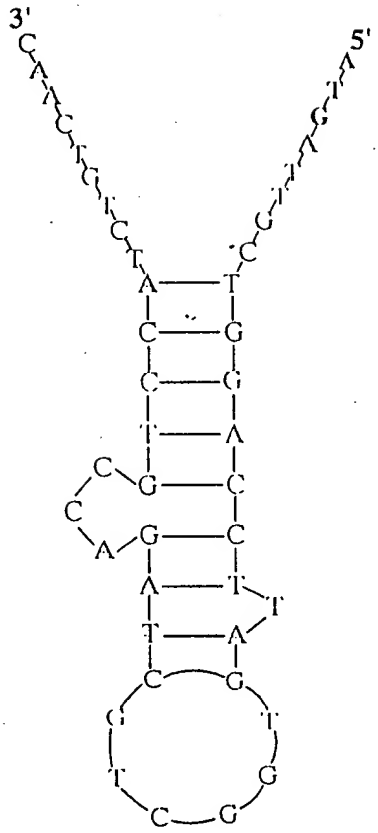
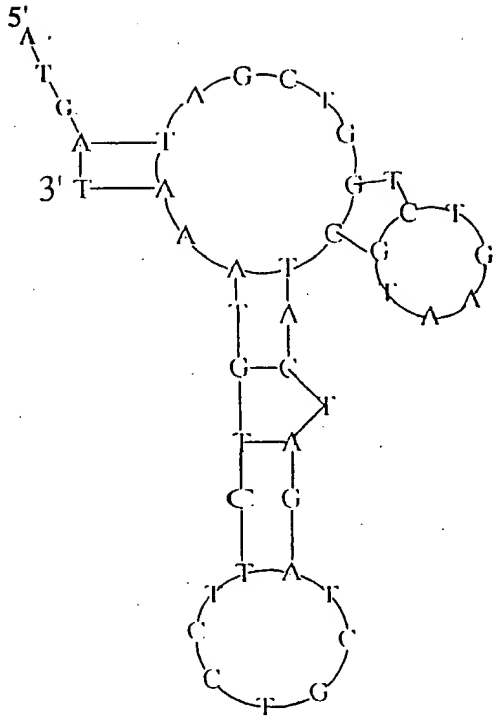
Fig. 12



△G=-10.3Kcal/mol

(A)



△G=-5.0Kcal/mol

(B)

Fig. 13

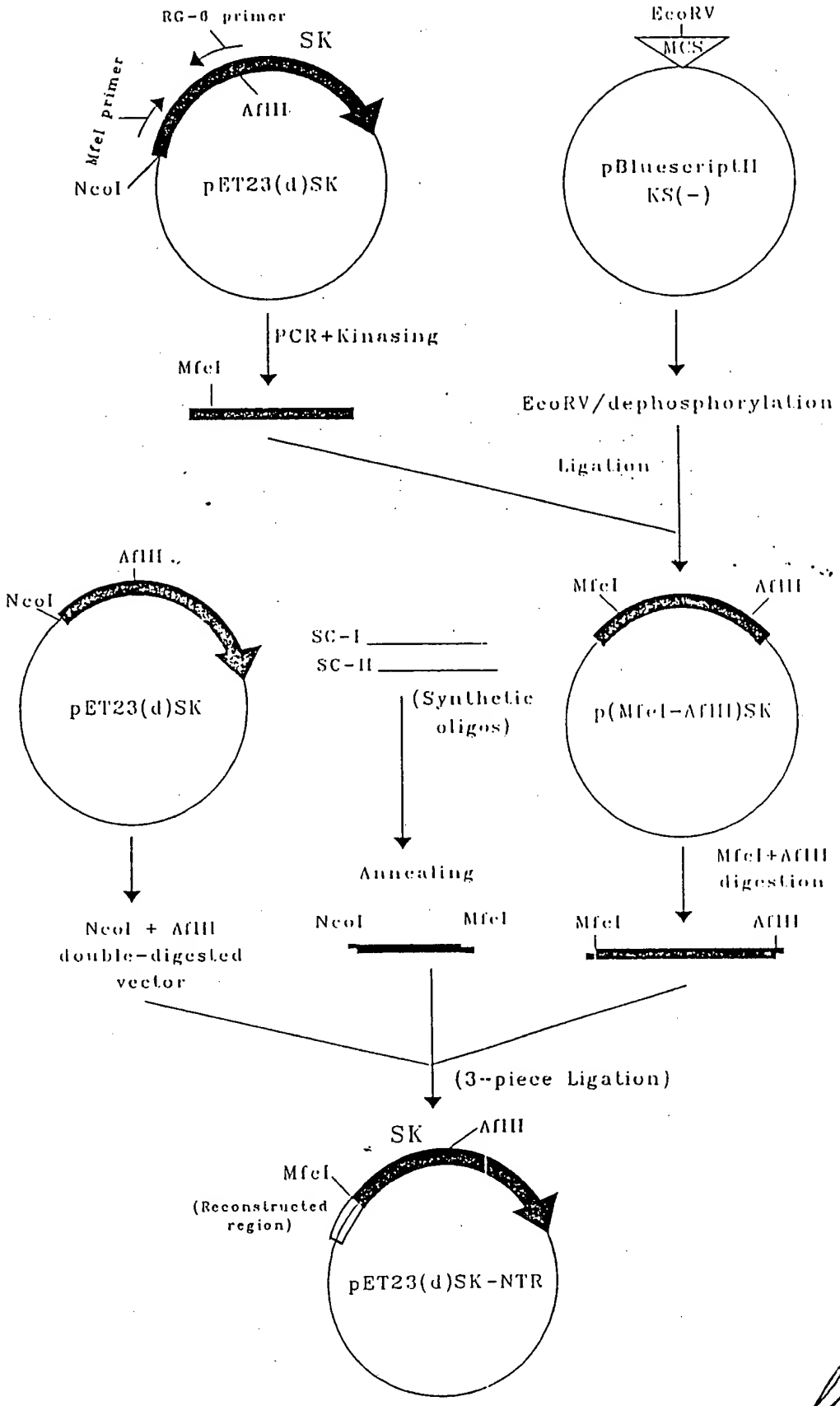

RG-0 primer
SK
MfeI primer
AflII
NcoI
pET23(d)SK
PCR+Kinasing
MfeI
EcoRV
MCS
pBlueseriptII
KS(-)
EcoRV/dephosphorylation
Ligation
AflII
NcoI
pET23(d)SK
SC-I
SC-II
(Synthetic
oligos)
MfeI
AflII
p(MfeI-AflII)SK
MfeI+AflII
digestion
Annealing
NcoI + AflII
double-digested
vector
NcoI
MfeI
MfeI
AflII
(3-piece Ligation)
SK
AflII
MfeI
(Reconstructed
region)
pET23(d)SK-NTR

Fig. 14

	10	20	30	40	50
	TAATACGACT	CACTATAGGG	AGACCACAAC	GGTTTCCCTC	TAGAAATAAT
51	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGATAGCTGG	TCCTGAATGG
101	CTACTAGATC	GTCCTTCTGT	AAATAACAGC	CAATTGGTTG	TTAGCGTTGC
151	TGGTACTGTT	GAGGGGACGA	ATCAAGACAT	TAGTCTTAAA	TTTTTTGAAA
201	TCGATCTAAC	ATCACGACCT	GCTCATGGAG	GAAAGACAGA	GCAAGGCTTA
251	AGTCCAAAAT	CAAAACCATT	TGCTACTGAT	AGTGGCGCGA	TGTCACATAA
301	ACTTGAGAAA	GCTGACTTAC	TAAAGGCTAT	TCAAGAACAA	TTGATCGCTA
351	ACGTCCACAG	TAACGACGAC	TACTTTGAGG	TCATTGATTT	TGCAAGCGAT
401	GCAACCATTA	CTGATCGAAA	CGGCAAGGTC	TACTTTGCTG	ACAAAGATGG
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501	ATGTGCGCGT	TAGACCATAT	AAAGAAAAAC	CAATACAAAA	CCAAGCGAAA
551	TCTGTTGATG	TGGAATATAC	TGTACAGTTT	ACTCCCTTAA	ACCCTGATGA
601	CGATTTCAGA	CCAGGTCTCA	AAGATACTAA	GCTATTGAAA	ACACTAGCTA
651	TCGGTGACAC	CATCACATCT	CAAGAATTAC	TAGCTCAAGC	ACAAAGCATT
701	TTAAACAAAA	ACCACCCAGG	CTATACGATT	TATGAACGTG	ACTCCTCAAT
751	CGTCACTCAT	GACAATGACA	TTTTCCGTAC	GATTTTACCA	ATGGATCAAG
801	AGTTTACTTA	CCGTGTTAAA	AATCGGGAAC	AAGCTTATAG	GATCAATAAA
851	AAATCTGGTC	TGAATGAAGA	AATAAACAAC	ACTGACCTGA	TCTCTGAGAA
901	ATATTACGTC	CTTAAAAAAG	GGGAAAAGCC	GTATGATCCC	TTTGATCGCA
951	GTCACTTGAA	ACTGTTCACC	ATCAAATACG	TTGATGTCGA	TACCAACGAA
1001	TTGCTAAAAA	GTGAGCAGCT	CTTAACAGCT	AGCGAACGTA	ACTTAGACTT
1051	CAGAGATTTA	TACGATCCTC	GTGATAAGGC	TAAACTACTC	TACAACAATC
1101	TCGATGCTTT	TGGTATTATG	GACTATACCT	TAACTGGAAA	AGTAGAGGAT
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1201	CGAAGGAGAG	AATGCTAGCT	ATCATTTAGC	CTATGATAAA	GATCGTTATA
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1301	ATACCTGATA	ACCCTAACGA	CAAATAA		

Fig. 15

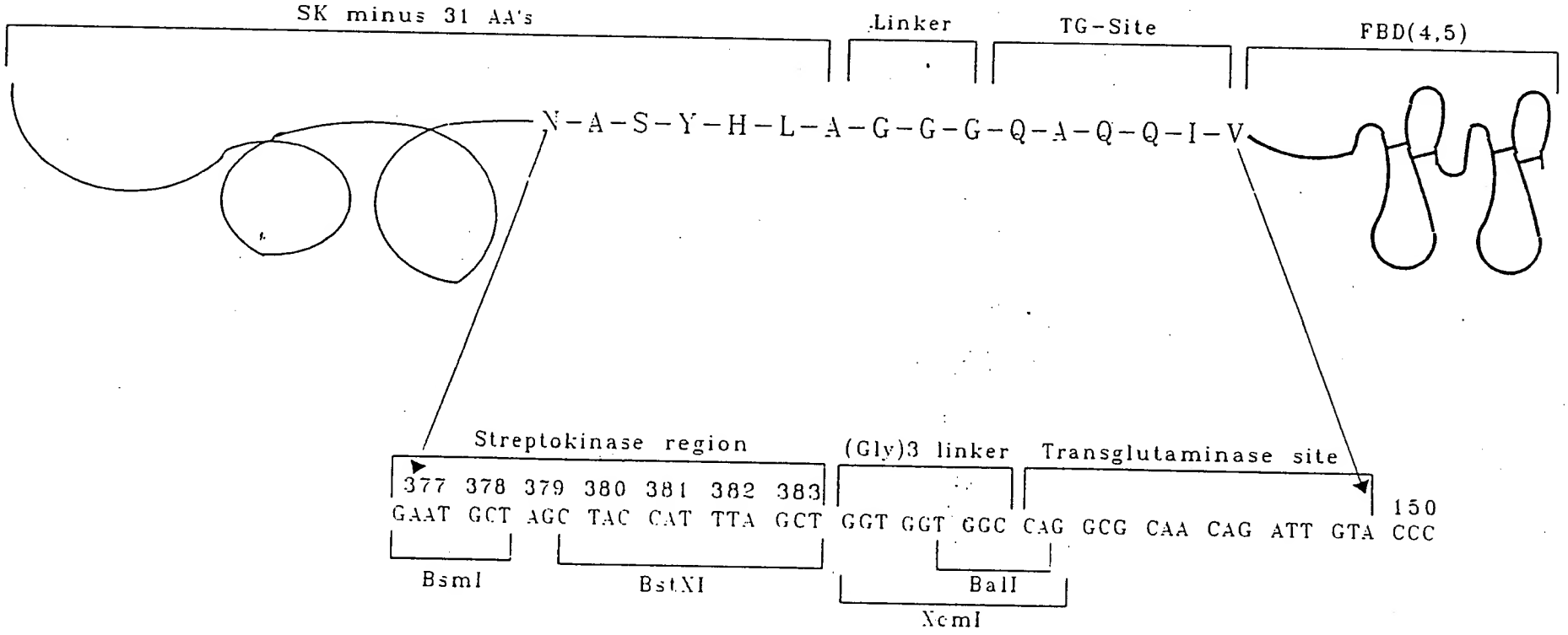
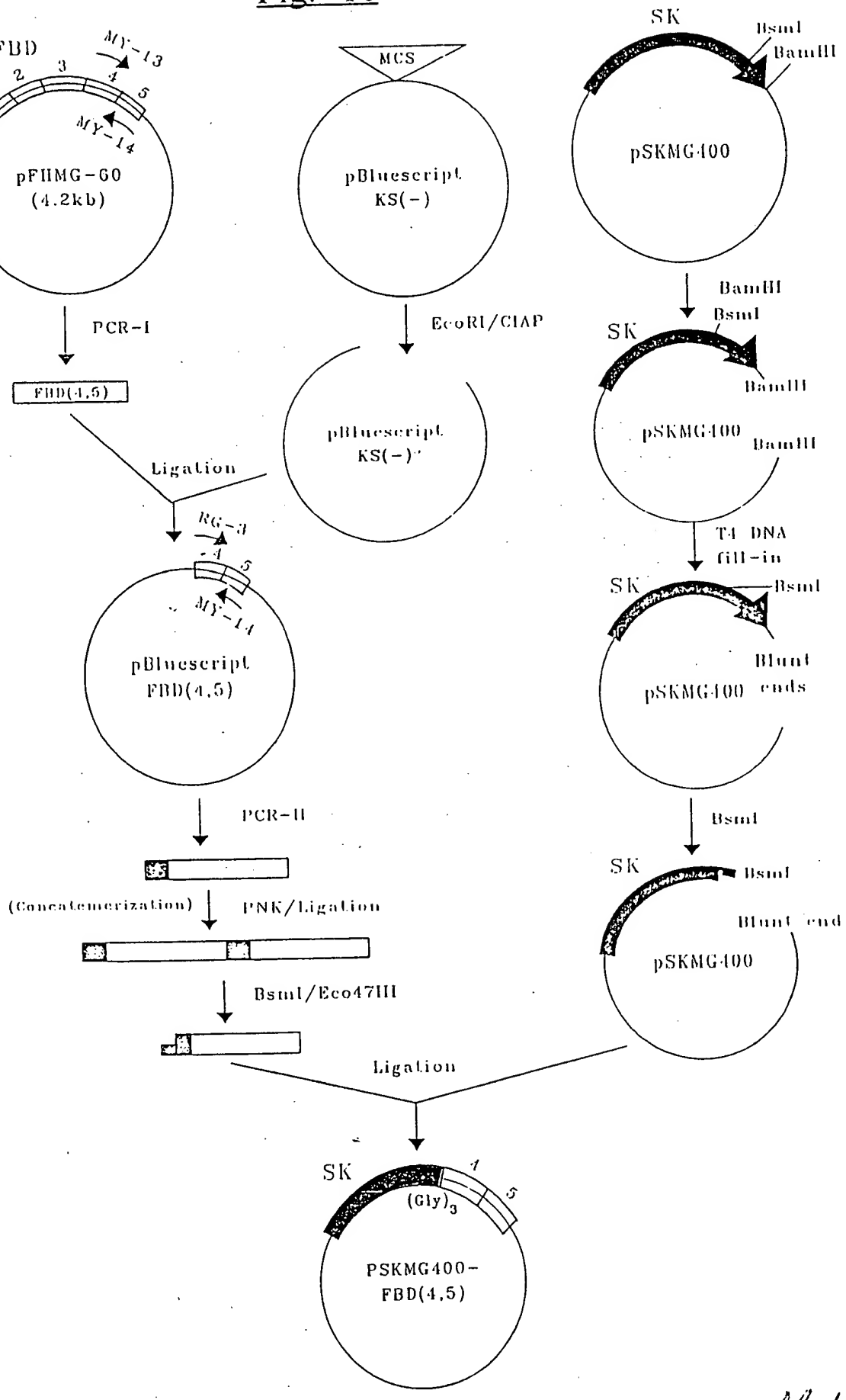

SK minus 31 AA's
Linker
TG-Site
FBD(4,5)
N-A-S-Y-H-L-A-G-G-G-Q-A-Q-Q-I-V
Streptokinase region
(Gly)3 linker
Transglutaminase site
377 378 379 380 381 382 383
GAAT GCT AGC TAC CAT TTA GCT GGT GGT GGC CAG GCG CAA CAG ATT GTA CCC
150
BsmI
BstXI
BalI
XcmI

Fig. 16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MY-13
1
2
3
4
5
MY-14
pFHMG-60
(4.2kb)
PCR-I
FBD(4,5)
Ligation
MCS
pBluescript
KS(-)
EcoRI/CIAP
pBluescript
KS(-)'
RG-3
4
5
MY-14
pBluescript
FBD(4,5)
PCR-II
(Concatemerization)
PNK/Ligation
BsmI/Eco47III
SK
BsmI
BamHI
pSKMG400
BamHI
SK
BsmI
BamHI
pSKMG400
BamHI
T4 DNA
fill-in
SK
BsmI
Blunt
pSKMG400 ends
BsmI
SK
BsmI
Blunt end
pSKMG400
Ligation
SK
1
5
(Gly)3
PSKMG400-
FBD(4,5)

Fig. 17a

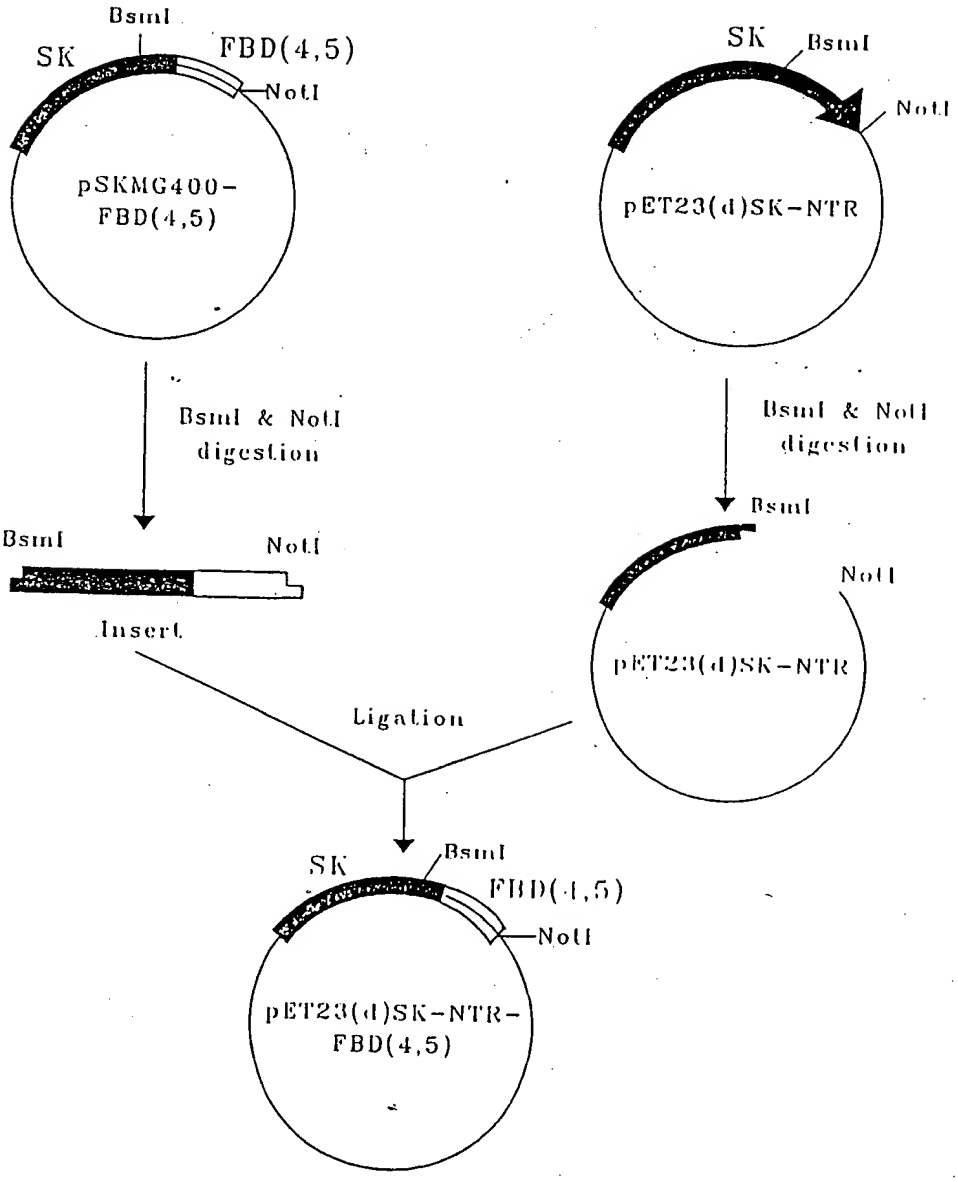
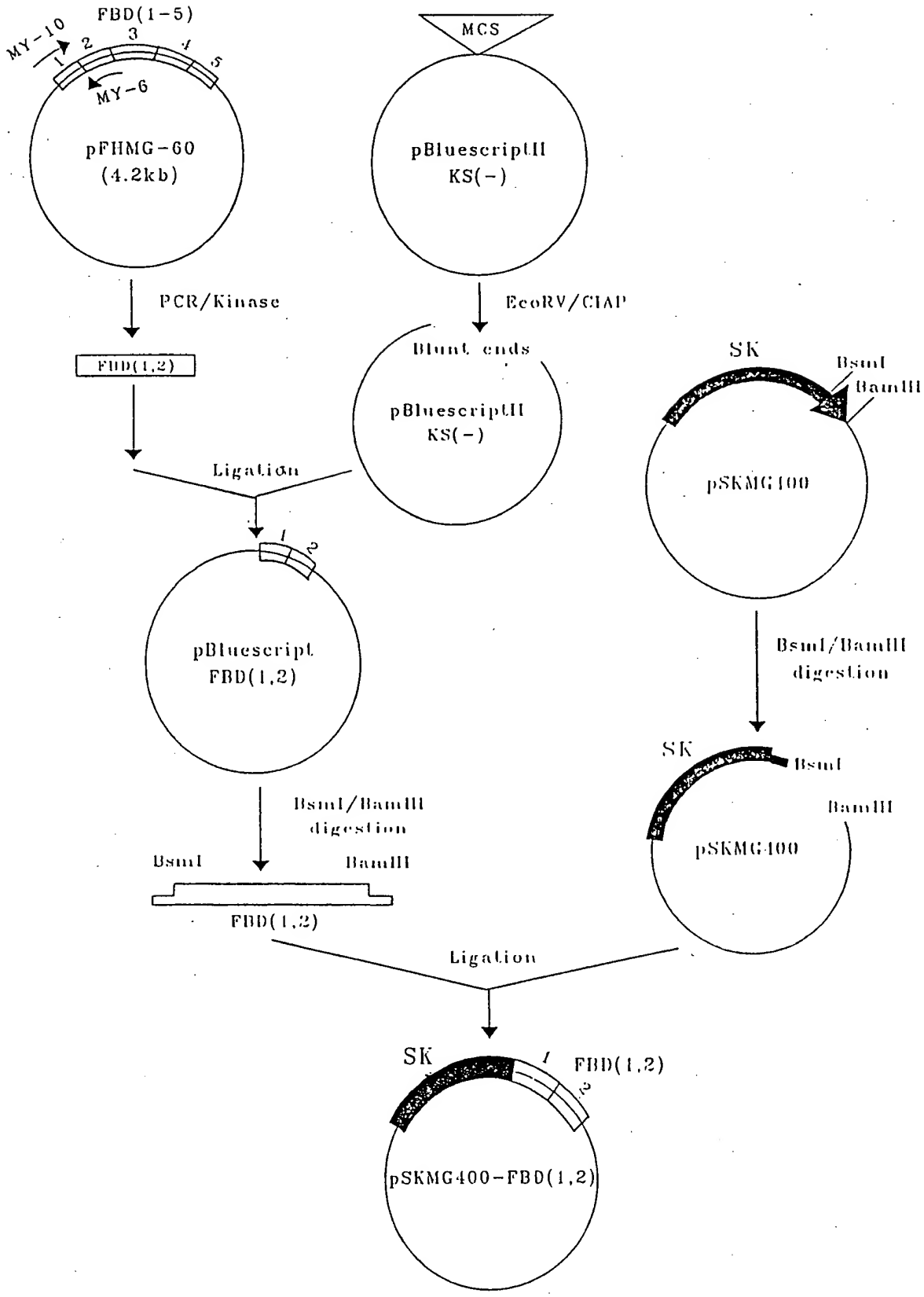

BsmI
SK
FBD(4,5)
NotI
pSKMG400-
FBD(4,5)
BsmI & NotI
digestion
BsmI
NotI
Insert
SK
BsmI
NotI
pET23(d)SK-NTR
BsmI & NotI
digestion
BsmI
NotI
pET23(d)SK-NTR
Ligation
SK
BsmI
FBD(4,5)
NotI
pET23(d)SK-NTR-
FBD(4,5)

Fig. 17b

	10	20	30	40	50
	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGATAGCTGG	TCCTGAATGG
51	CTACTAGATC	GTCCTTCTGT	AAATAACAGC	CAATTGGTTG	TTAGCGTTGC
101	TGGTACTGTT	GAGGGGACGA	ATCAAGACAT	TAGTCTTAAA	TTTTTTGAAA
151	TCGATCTAAC	ATCACGACCT	GCTCATGGAG	GAAAGACAGA	GCAAGGCTTA
201	AGTCCAAAAT	CAAAACCATT	TGCTACTGAT	AGTGGCGCGA	TGTCACATAA
251	ACTTGAGAAA	GCTGACTTAC	TAAAGGCTAT	TCAAGAACAA	TTGATCGCTA
301	ACGTCCACAG	TAACGACGAC	TACTTTGAGG	TCATTGATTT	TGCAAGCGAT
351	GCAACCATTA	CTGATCGAAA	CGGCAAGGTC	TACTTTGCTG	ACAAAGATGG
401	TTCGGTAACC	TTGCCGACCC	AACCTGTCCA	AGAATTTTTG	CTAAGCGGAC
451	ATGTGCGCGT	TAGACCATAT	AAAGAAAAAC	CAATACAAAA	CCAAGCGAAA
501	TCTGTTGATG	TGGAATATAC	TGTACAGTTT	ACTCCCTTAA	ACCCTGATGA
551	CGATTTCAGA	CCAGGTCTCA	AAGATACTAA	GCTATTGAAA	ACACTAGCTA
601	TCGGTGACAC	CATCACATCT	CAAGAATTAC	TAGCTCAAGC	ACAAAGCATT
651	TTAAACAAAA	ACCACCCAGG	CTATACGATT	TATGAACGTG	ACTCCTCAAT
701	CGTCACTCAT	GACAATGACA	TTTTCCGTAC	GATTTTACCA	ATGGATCAAG
751	AGTTTACTTA	CCGTGTTAAA	AATCGGGAAC	AAGCTTATAG	GATCAATAAA
801	AAATCTGGTC	TGAATGAAGA	AATAAACAAC	ACTGACCTGA	TCTCTGAGAA
851	ATATTACGTC	CTTAAAAAAG	GGGAAAAGCC	GTATGATCCC	TTTGATCGCA
901	GTCACTTGAA	ACTGTTCACC	ATCAAATACG	TTGATGTCGA	TACCAACGAA
951	TTGCTAAAAA	GTGAGCAGCT	CTTAACAGCT	AGCGAACGTA	ACTTAGACTT
1001	CAGAGATTTA	TACGATCCTC	GTGATAAGGC	TAAACTACTC	TACAACAATC
1051	TCGATGCTTT	TGGTATTATG	GACTATACCT	TAACTGGAAA	AGTAGAGGAT
1101	AATCACGATG	ACACCAACCG	TATCATAACC	GTTTATATGG	GCAAGCGACC
1151	CGAAGGAGAG	AATGCTAGCT	ACCATTTAGC	TGGTGGTGGC	CAGGCGCAAC
1201	AGATTGTACC	CATAGCTGAG	AAGTGTTTTG	ATCATGCTGC	TGGGACTTCC
1251	TATGTGGTCG	GAGAAACGTG	GGAGAAGCCC	TACCAAGGCT	GGATGATGGT
1301	AGATTGTACT	TGCCTGGGAG	AAGGCAGCGG	ACGCATCACT	TGCACTTCTA
1351	GAAATAGATG	CAACGATCAG	GACACAAGGA	CATCCTATAG	AATTGGAGAC
1401	ACCTGGAGCA	AGAAGGATAA	TCGAGGAAAC	CTGCTCCAGT	GCATCTGCAC
1451	AGGCAACGGC	CGAGGAGAGT	GGAAGTGTGA	GAGGCACACC	TCTGTGCAGA
1501	CCACATCGAG	CGGATCTGGC	CCCTTCACCG	ATGTTCGTTA	G

Fig. 18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MY-6
pFHMG-60
(4.2kb)
MCS
pBluescriptII
KS(-)
PCR/Kinase
EcoRV/CIAP
FBD(1,2)
Blunt ends
pBluescriptII
KS(-)
Ligation
SK
BsmI
BamHI
pSKMG100
pBluescript
FBD(1,2)
BsmI/BamHI
digestion
BsmI
BamHI
FBD(1,2)
SK
BsmI
BamHI
pSKMG400
Ligation
SK
FBD(1,2)
pSKMG400-FBD(1,2)

Fig. 19a

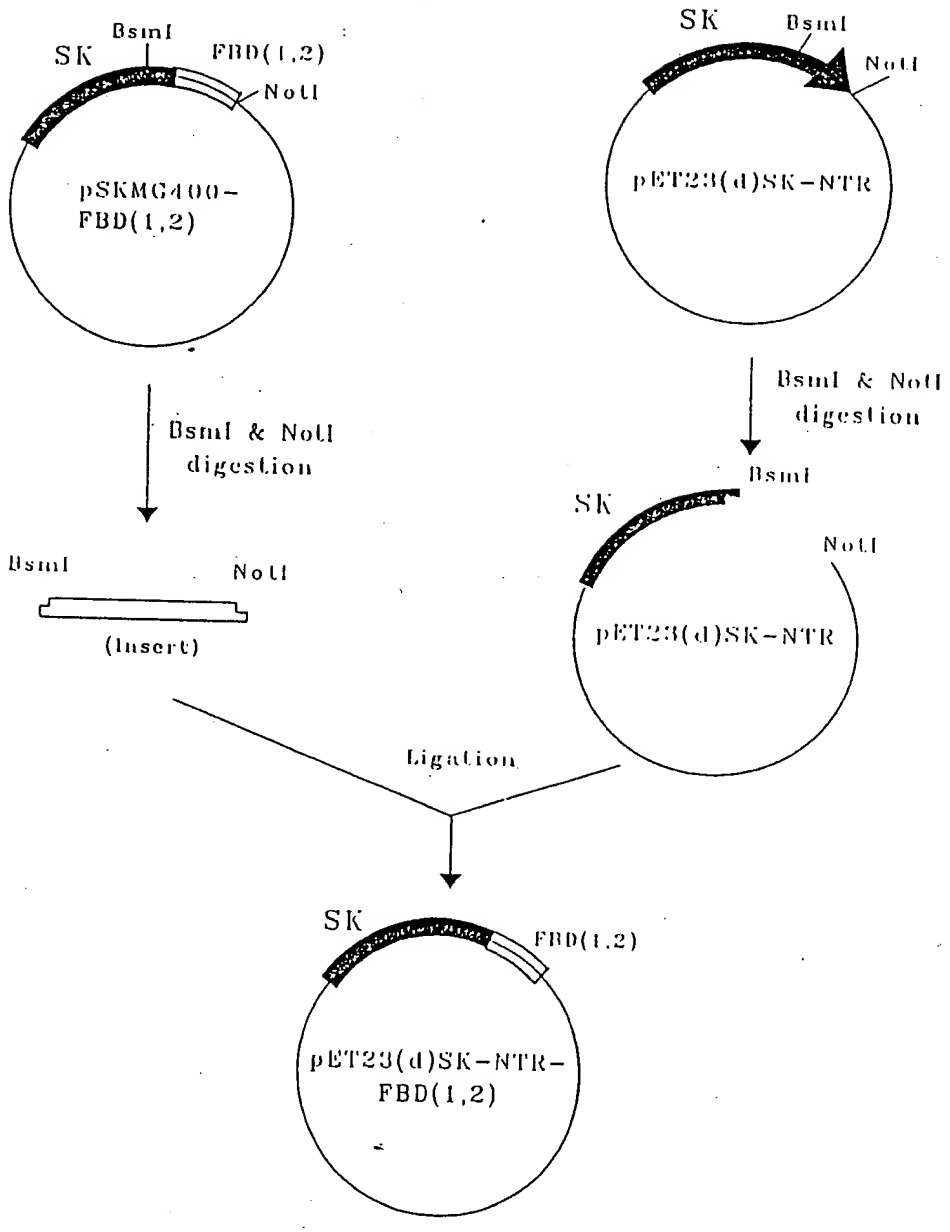
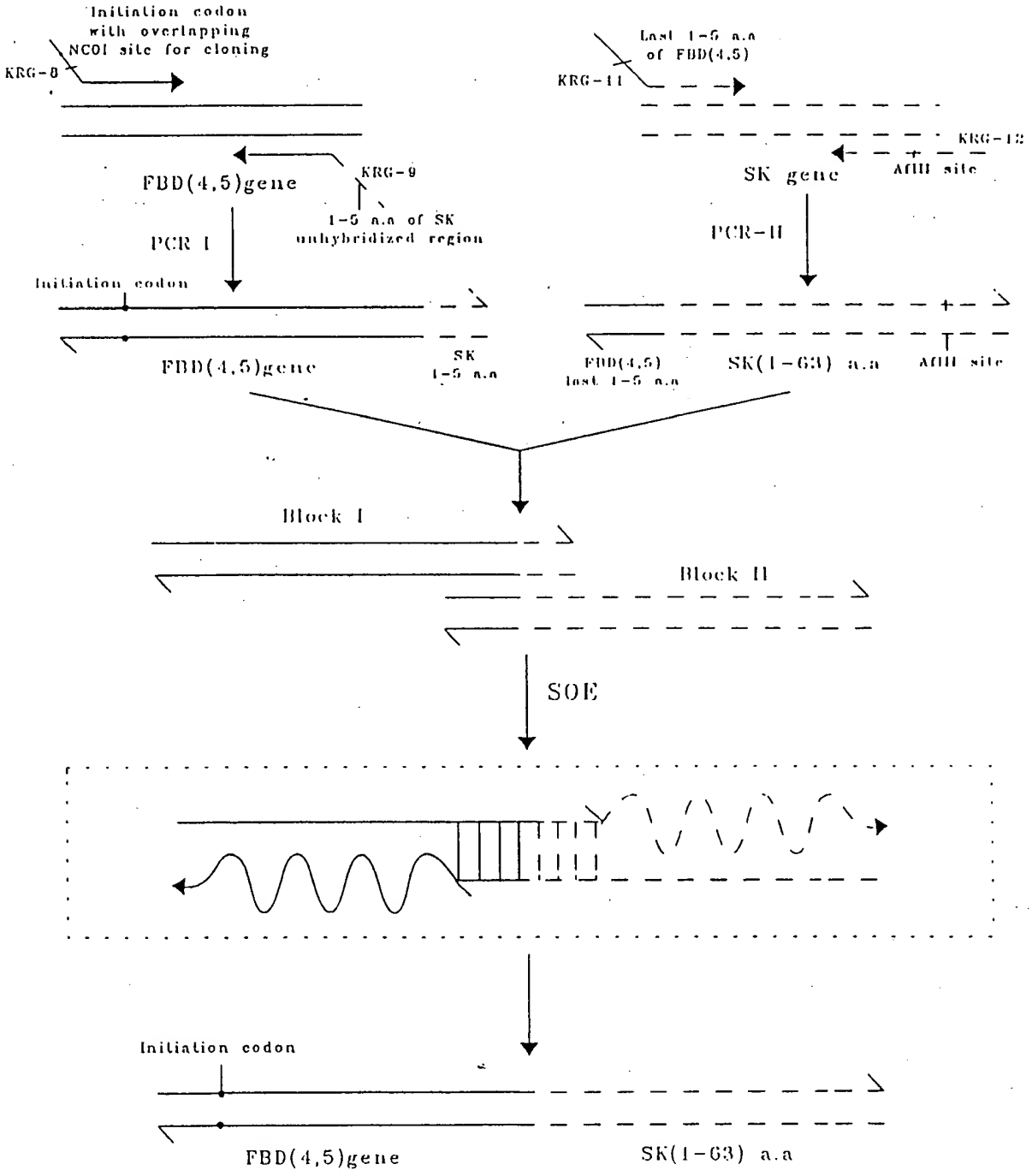

SK
BsmI
FBD(1,2)
NotI
pSKMG400-FBD(1,2)
SK
BsmI
NotI
pET23(d)SK-NTR
BsmI & NotI digestion
BsmI & NotI digestion
BsmI
NotI
(Insert)
SK
BsmI
NotI
pET23(d)SK-NTR
Ligation
SK
FBD(1,2)
pET23(d)SK-NTR-FBD(1,2)

Fig. 19b

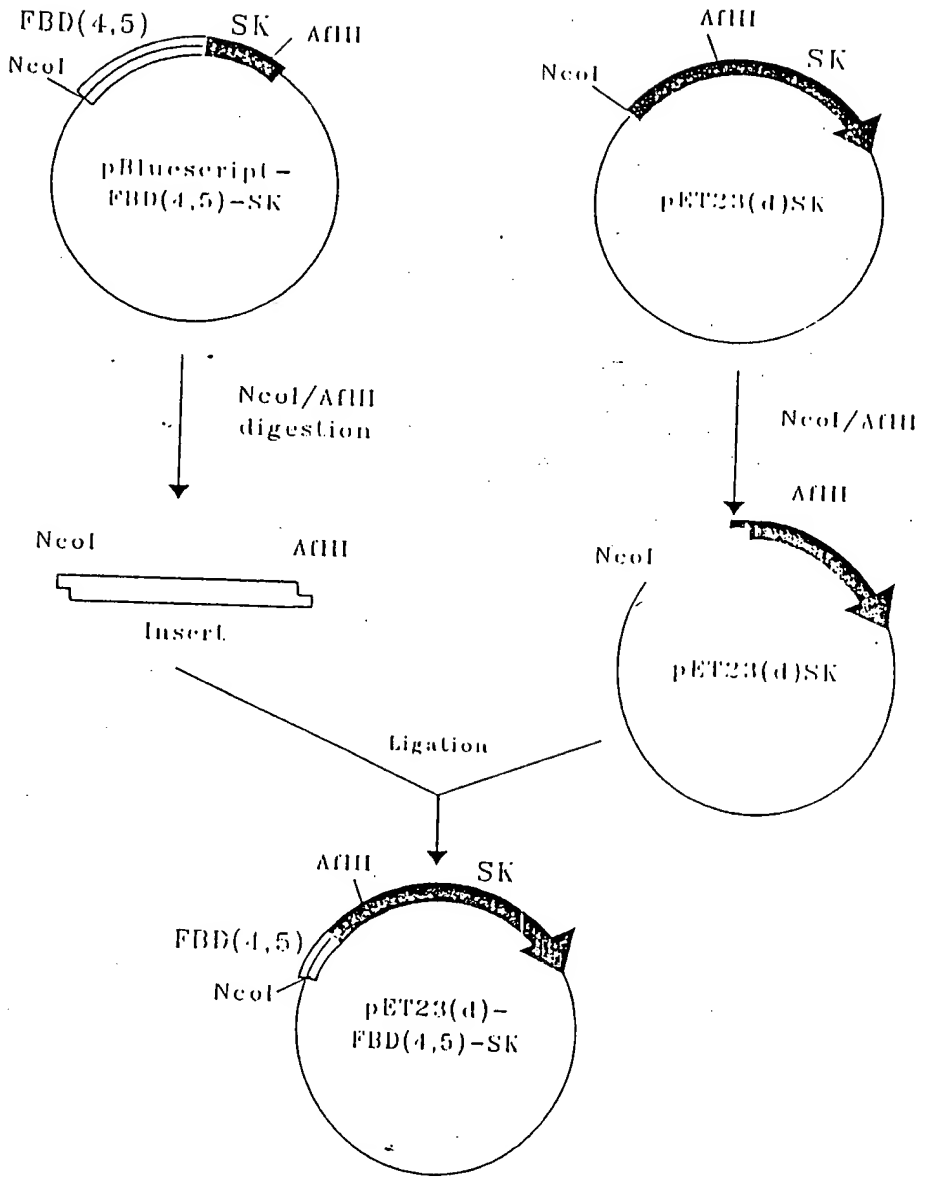
	10	20	30	40	50
	GCAACCCCGC	CAGCCTAGCC	GGGTCCTCAA	CGACAGGAGC	ACGATCATGC
51	GCACCCGTGG	CCAGGACCCA	ACGCTGCCCG	AGATCTCGAT	CCCGCGAAAT
101	TAATACGACT	CACTATAGGG	AGACCACAAC	GGTTTCCCTC	TAGAAATAAT
151	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGATTGCTGG	ACCTGAGTGG
201	CTGCTAGACC	GTCCATCTGT	CAACAACAGC	CAATTGGTTG	TTAGCGTTGC
251	TGGTACTGTT	GAGGGGACGA	ATCAAGACAT	TAGTCTTAAA	TTTTTTGAAA
301	TCGATCTAAC	ATCACGACCT	GCTCATGGAG	GAAAGACAGA	GCAAGGCTTA
351	AGTCCAAAAT	CAAAACCATT	TGCTACTGAT	AGTGGCGCGA	TGTCACATAA
401	ACTTGAGAAA	GCTGACTTAC	TAAAGGCTAT	TCAAGAACAA	TTGATCGCTA
451	ACGTCCACAG	TAACGACGAC	TACTTTGAGG	TCATTGATTT	TGCAAGCGAT
501	GCAACCATTA	CTGATCGAAA	CGGCAAGGTC	TACTTTGCTG	ACAAAGATGG
551	TTCGGTAACC	TTGCCGACCC	AACCTGTCCA	AGAATTTTTG	CTAAGCGGAC
601	ATGTGCGCGT	TAGACCATAT	AAAGAAAAAC	CAATACAAAA	CCAAGCGAAA
651	TCTGTTGATG	TGGAATATAC	TGTACAGTTT	ACTCCCTTAA	ACCCTGATGA
701	CGATTTCAGA	CCAGGTCTCA	AAGATACTAA	GCTATTGAAA	ACACTAGCTA
751	TCGGTGACAC	CATCACATCT	CAAGAATTAC	TAGCTCAAGC	ACAAAGCATT
801	TTAAACAAAA	ACCACCCAGG	CTATACGATT	TATGAACGTG	ACTCCTCAAT
851	CGTCACTCAT	GACAATGACA	TTTTCCGTAC	GATTTTACCA	ATGGATCAAG
901	AGTTTACTTA	CCGTGTTAAA	AATCGGGAAC	AAGCTTATAG	GATCAATAAA
951	AAATCTGGTC	TGAATGAAGA	AATAAACAAC	ACTGACCTGA	TCTCTGAGAA
1001	ATATTACGTC	CTTAAAAAAG	GGGAAAAGCC	GTATGATCCC	TTTGATCGCA
1051	GTCACTTGAA	ACTGTTCACC	ATCAAATACG	TTGATGTCGA	TACCAACGAA
1101	TTGCTAAAAA	GTGAGCAGCT	CTTAACAGCT	AGCGAACGTA	ACTTAGACTT
1151	CAGAGATTTA	TACGATCCTC	GTGATAAGGC	TAAACTACTC	TACAACAATC
1201	TCGATGCTTT	TGGTATTATG	GACTATACCT	TAACTGGAAA	AGTAGAGGAT
1251	AATCACGATG	ACACCAACCG	TATCATAACC	GTTTATATGG	GCAAGCGACC
1301	CGAAGGAGAG	AATGCTAGCT	ATCATTTAGC	CGGTGGTGGT	CAGGCGCAGC
1351	AAATGGTTCA	GCCCCAGTCC	CCGGTGGCTG	TCAGTCAAAG	CAAGCCCGGT
1401	TGTTATGACA	ATGGAAAACA	CTATCAGATA	AATCAACAGT	GGGAGCGGAC
1451	CTACCTAGGT	AATGTGTTGG	TTTGTACTTG	TTATGGAGGA	AGCCGAGGTT
1501	TTAACTGCGA	AAGTAAACCT	GAAGCTGAAG	AGACTTGCTT	TGACAAGTAC
1551	ACTGGGAACA	CTTACCGAGT	GGGTGACACT	TATGAGCGTC	CTAAAGACTC
1601	CATGATCTGG	GACTGTACCT	GCATCGGGGC	TGGGCGAGGG	AGAATAAGCT
1651	GTACCATCTA	A			

Fig. 20



Recombinant Product

Fig. 21a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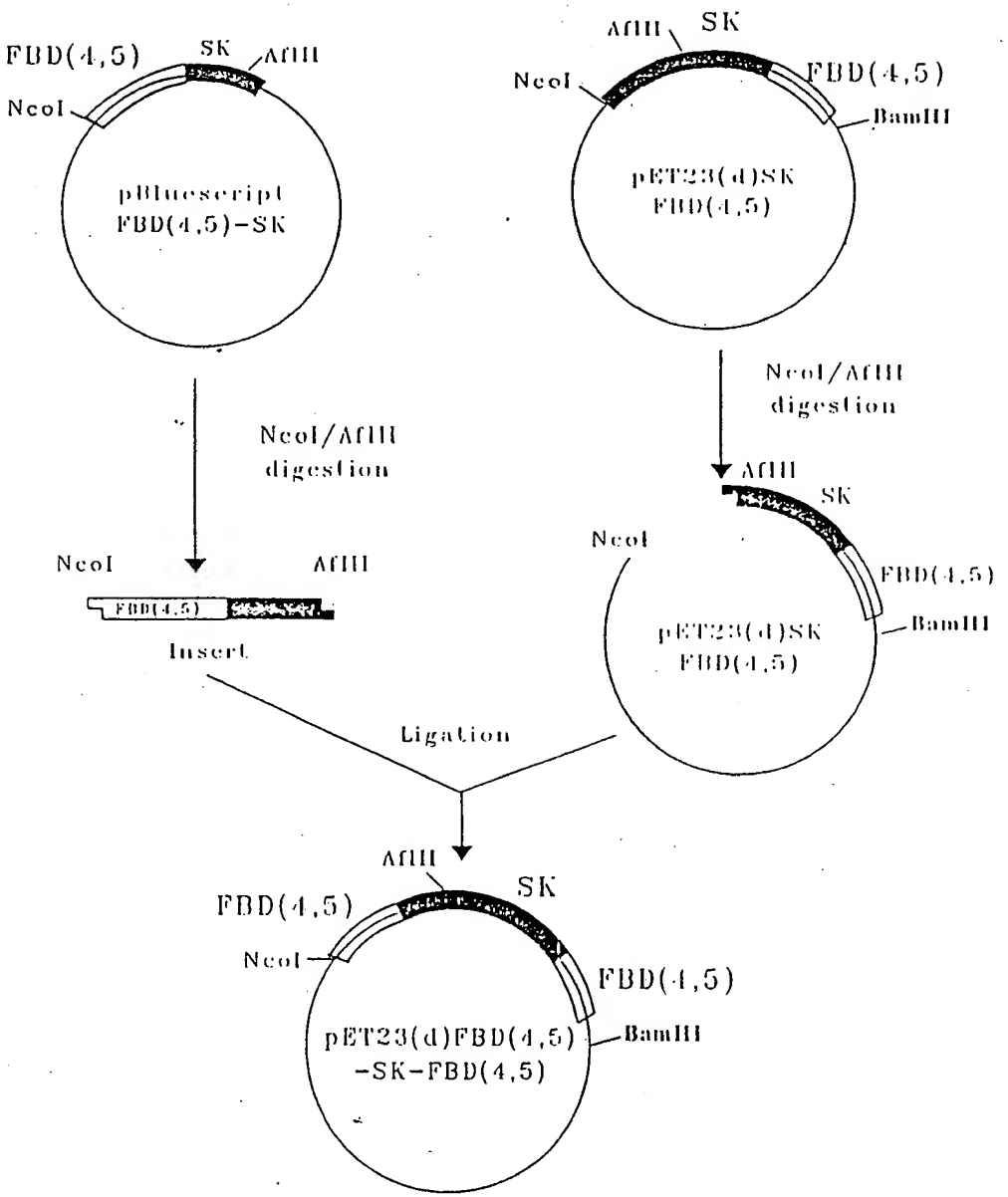
AflII
NcoI
pBluescript-
FBD(4,5)-SK
NcoI/AflII
digestion
NcoI
AflII
Insert
AflII
SK
NcoI
pET23(d)SK
NcoI/AflII
AflII
NcoI
pET23(d)SK
Ligation
AflII
SK
FBD(4,5)
NcoI
pET23(d)-
FBD(4,5)-SK

Fig. 21b

	10	20	30	40	50
1	TCGCTTCACG	TTCGCTCGCG	TATCGGTGAT	TCATTCTGCT	AACCAGTAAG
51	GCAACCCCGC	CAGCCTAGCC	GGGTCCTCAA	CGACAGGAGC	ACGATCATGC
101	GCACCCGTGG	CCAGGACCCA	ACGCTGCCCG	AGATCTCGAT	CCCGCGAAAT
151	TAATACGACT	CACTATAGGG	AGACCACAAC	GGTTTCCCTC	TAGAAATAAT
201	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGGTGCAAGC	ACAACAGATT
251	GTACCCATAG	CTGAGAAGTG	TTTTGATCAT	GCTGCTGGGA	CTTCCTATGT
301	GGTCGGAGAA	ACGTGGGAGA	AGGCAGCGGA	CGCATCACTT	GCACTTCTAG
351	AAATAGATGC	AACGATCAGG	ACACAAGGAC	ATCCTATAGA	ATTGGAGACA
401	CCTGGAGCAA	GAAGGATAAT	CGAGGAAACC	TGCTCCAGTG	CATCTGCACA
451	GGCAACGGCC	GAGGAGAGTG	GAAGTGTGAG	AGGCACACCT	CTGTGCAGAC
501	CACATCGAGC	GGATCTGGCC	CCTTCACCGA	TGTTCGTATT	GCTGGACCTG
551	AGTGGCTGCT	AGACCGTCCA	TCTGTCAACA	ACAGCCAATT	GGTTGTTAGC
601	GTTGCTGGTA	CTGTTGAGGG	GACGAATCAA	GACATTAGTC	TTAAATTTTT
651	TGAAATCGAT	CTAACATCAC	GACCTGCTCA	TGGAGGAAAG	ACAGAGCAAG
701	GCTTAAGTCC	AAAATCAAAA	CCATTTGCTA	CTGATAGTGG	CGCGATGTCA
751	CATAAACTTG	AGAAAGCTGA	CTTACTAAAG	GCTATTCAAG	AACAATTGAT
801	CGCTAACGTC	CACAGTAACG	ACGACTACTT	TGAGGTCATT	GATTTTGCAA
851	GCGATGCAAC	CATTACTGAT	CGAAACGGCA	AGGTCTACTT	TGCTGACAAA
901	GATGGTTCGG	TAACCTTGCC	GACCCAACCT	GTCCAAGAAT	TTTTGCTAAG
951	CGGACATGTG	CGCGTTAGAC	CATATAAAGA	AAAACCAATA	CAAAACCAAG
1001	CGAAATCTGT	TGATGTGGAA	TATACTGTAC	AGTTTACTCC	CTTAAACCCT
1051	GATGACGATT	TCAGACCAGG	TCTCAAAGAT	ACTAAGCTAT	TGAAAACACT
1101	AGCTATCGGT	GACACCATCA	CATCTCAAGA	ATTACTAGCT	CAAGCACAAA
1151	GCATTTTAAA	CAAAAACCAC	CCAGGCTATA	CGATTTATGA	ACGTGACTCC
1201	TCAATCGTCA	CTCATGACAA	TGACATTTTC	CGTACGATTT	TACCAATGGA
1251	TCAAGAGTTT	ACTTACCGTG	TTAAAAATCG	GGAACAAGCT	TATAGGATCA
1301	ATAAAAAATC	TGGTCTGAAT	GAAGAAATAA	ACAACACTGA	CCTGATCTCT
1351	GAGAAATATT	ACGTCCTTAA	AAAAGGGGAA	AAGCCGTATG	ATCCCTTTGA
1401	TCGCAGTCAC	TTGAAACTGT	TCACCATCAA	ATACGTTGAT	GTCGATACCA
1451	ACGAATTGCT	AAAAAGTGAG	CAGCTCTTAA	CAGCTAGCGA	ACGTAACTTA
1501	GACTTCAGAG	ATTTATACGA	TCCTCGTGAT	AAGGCTAAAC	TACTCTACAA
1551	CAATCTCGAT	GCTTTTGGTA	TTATGGACTA	TACCTTAACT	GGAAAAGTAG
1601	AGGATAATCA	CGATGACACC	AACCGTATCA	TAACCGTTTA	TATGGGCAAG
1651	CGACCCGAAG	GAGAGAATGC	TAGCTATCAT	TTAGCCTATG	ATAAAGATCG
1701	TTATACCGAA	GAAGAACGAG	AAGTTTACAG	CTACCTGCGT	TATACAGGGA
1751	CACCTATACC	TGATAACCCT	AACGACAAAT	AA	

Fig. 22a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FBD(4,5)-SK
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SK
NcoI
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BamHI
pET23(d)SK
FBD(4,5)
NcoI/AflII
digestion
NcoI/AflII
digestion
NcoI
AflII
FBD(4,5)
Insert
AflII
SK
NcoI
FBD(4,5)
BamHI
pET23(d)SK
FBD(4,5)
Ligation
AflII
FBD(4,5)
SK
NcoI
FBD(4,5)
BamHI
pET23(d)FBD(4,5)
-SK-FBD(4,5)

Fig. 22b

	10	20	30	40	50
1	CGAAGACCAT	TCATGTTGTT	GCTCAGGTCG	CAGACGTTTT	GCAGCAGCAG
51	TCGCTTCACG	TTCGCTCGCG	TATCGGTGAT	TCATTCTGCT	AACCAGTAAG
101	GCAACCCCGC	CAGCCTAGCC	GGGTCCTCAA	CGACAGGAGC	ACGATCATGC
151	GCACCCGTGG	CCAGGACCCA	ACGCTGCCCG	AGATCTCGAT	CCCGCGAAAT
201	TAATACGACT	CACTATAGGG	AGACCACAAC	GGTTTCCCTC	TAGAAATAAT
251	TTTGTTTAAC	TTTAAGAAGG	AGATATACCA	TGGTGCAAGC	ACAACAGATT
301	GTACCCATAG	CTGAGAAGTG	TTTTGATCAT	GCTGCTGGGA	CTTCCTATGT
351	GGTCGGAGAA	ACGTGGGAGA	AGGCAGCGGA	CGCATCACTT	GCACTTCTAG
401	AAATAGATGC	AACGATCAGG	ACACAAGGAC	ATCCTATAGA	ATTGGAGACA
451	CCTGGAGCAA	GAAGGATAAT	CGAGGAAACC	TGCTCCAGTG	CATCTGCACA
501	GGCAACGGCC	GAGGAGAGTG	GAAGTGTGAG	AGGCACACCT	CTGTGCAGAC
551	CACATCGAGC	GGATCTGGCC	CCTTCACCGA	TGTTCGTATT	GCTGGACCTG
601	AGTGGCTGCT	AGACCGTCCA	TCTGTCAACA	ACAGCCAATT	GGTTGTTAGC
651	GTTGCTGGTA	CTGTTGAGGG	GACGAATCAA	GACATTAGTC	TTAAATTTTT
701	TGAAATCGAT	CTAACATCAC	GACCTGCTCA	TGGAGGAAAG	ACAGAGCAAG
751	GCTTAAGTCC	AAAATCAAAA	CCATTTGCTA	CTGATAGTGG	CGCGATGTCA
801	CATAAACTTG	AGAAAGCTGA	CTTACTAAAG	GCTATTCAAG	AACAATTGAT
851	CGCTAACGTC	CACAGTAACG	ACGACTACTT	TGAGGTCATT	GATTTTGCAA
901	GCGATGCAAC	CATTACTGAT	CGAAACGGCA	AGGTCTACTT	TGCTGACAAA
951	GATGGTTCGG	TAACCTTGCC	GACCCAACCT	GTCCAAGAAT	TTTTGCTAAG
1001	CGGACATGTG	CGCGTTAGAC	CATATAAAGA	AAAACCAATA	CAAAACCAAG
1051	CGAAATCTGT	TGATGTGGAA	TATACTGTAC	AGTTTACTCC	CTTAAACCCT
1101	GATGACGATT	TCAGACCAGG	TCTCAAAGAT	ACTAAGCTAT	TGAAAACACT
1151	AGCTATCGGT	GACACCATCA	CATCTCAAGA	ATTACTAGCT	CAAGCACAAA
1201	GCATTTTAAA	CAAAAACCAC	CCAGGCTATA	CGATTTATGA	ACGTGACTCC
1251	TCAATCGTCA	CTCATGACAA	TGACATTTTC	CGTACGATTT	TACCAATGGA
1301	TCAAGAGTTT	ACTTACCGTG	TTAAAAATCG	GGAACAAGCT	TATAGGATCA
1351	ATAAAAAATC	TGGTCTGAAT	GAAGAAATAA	ACAACACTGA	CCTGATCTCT
1401	GAGAAATATT	ACGTCCTTAA	AAAAGGGGAA	AAGCCGTATG	ATCCCTTTGA
1451	TCGCAGTCAC	TTGAAACTGT	TCACCATCAA	ATACGTTGAT	GTCGATACCA
1501	ACGAATTGCT	AAAAAGTGAG	CAGCTCTTAA	CAGCTAGCGA	ACGTAACTTA
1551	GACTTCAGAG	ATTTATACGA	TCCTCGTGAT	AAGGCTAAAC	TACTCTACAA
1601	CAATCTCGAT	GCTTTTGGTA	TTATGGACTA	TACCTTAACT	GGAAAAGTAG
1651	AGGATAATCA	CGATGACACC	AACCGTATCA	TAACCGTTTA	TATGGGCAAG
1701	CGACCCGAAG	GAGAGAATGC	TAGCTACCAT	TTAGCTGGTG	GTGGCCAGGC
1751	GCAACAGATT	GTACCCATAG	CTGAGAAGTG	TTTTGATCAT	GCTGCTGGGA
1801	CTTCCTATGT	GGTCGGAGAA	ACGTGGGAGA	AGCCCTACCA	AGGCTGGATG
1851	ATGGTAGATT	GTACTTGCCT	GGGAGAAGGC	AGCGGACGCA	TCACTTGCAC
1901	TTCTAGAAAT	AGATGCAACG	ATCAGGACAC	AAGGACATCC	TATAGAATTG
1951	GAGACACCTG	GAGCAAGAAG	GATAATCGAG	GAAACCTGCT	CCAGTGCATC
2001	TGCACAGGCA	ACGGCCGAGG	AGAGTGGAAG	TGTGAGAGGC	ACACCTCTGT
2051	GCAGACCACA	TCGAGCGGAT	CTGGCCCCTT	CACCGATGTT	CGTTAG

Fig. 23

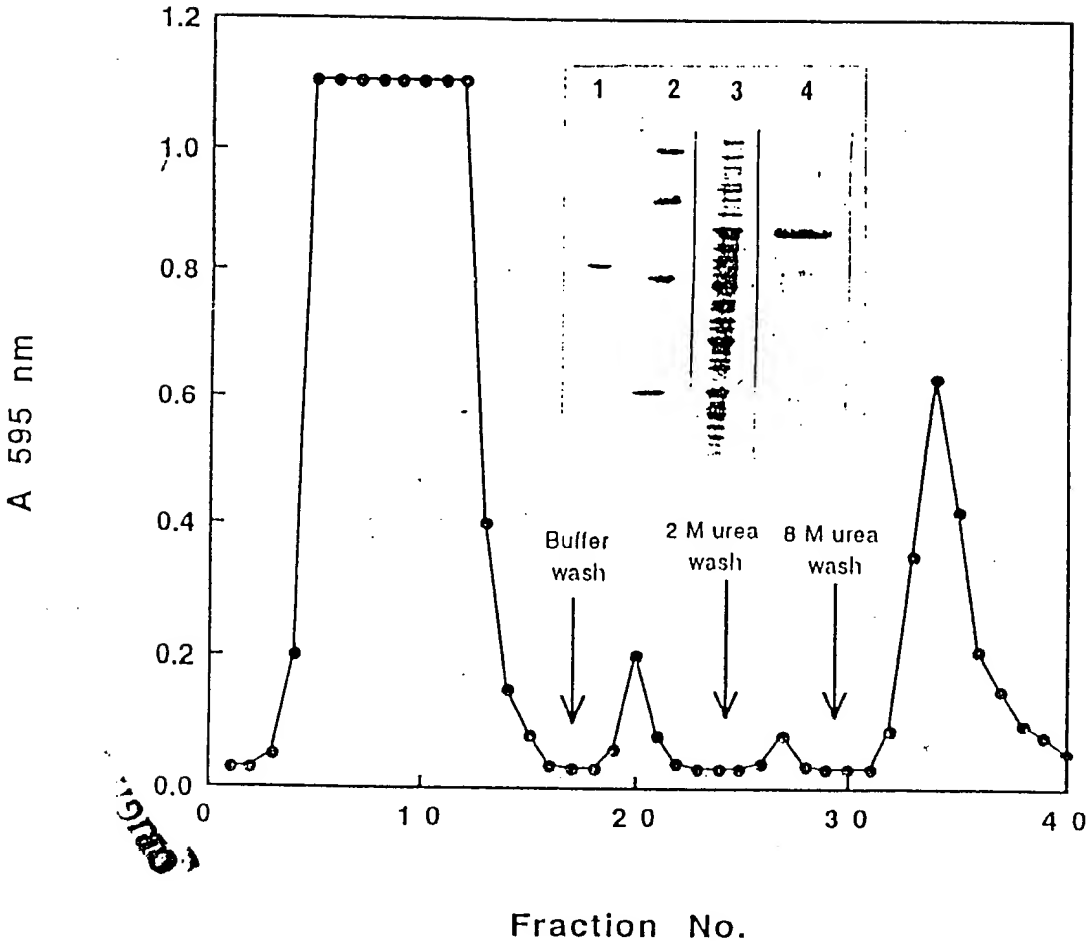

Fig. 24